REMARKS

Claims 19-27 and 29-35 are pending. Applicants have amended claims 1-35 to recite "pharmaceutical preparation," support for which may be found in the originally filed specification *inter alia* at page 1, line 6. Claims 23 and 30 have also been amended to delete "and alcohol." New claims 36-53 have been added, support for which may be found in the originally filed specification *inter alia* at page 1, lines 6-19. These amendments are made to clarify the present invention and not intended to narrow or limit the scope of the present invention. No new matter has been added. Applicants respectfully request entry of the present amendment. Accordingly, claims 19-27 and 29-53 will be pending.

Claim Rejections under 35 U.S.C. § 103(a)

The Examiner has maintained the rejection of Claims 19-35 under 35 U.S.C. § 103(a) as being unpatentable over Gierskcky et al. U.S. Patent No. 6,034,267 ("Gierskcky").

The Examiner states that modification of proportions and ranges is not patentable unless there is a showing of criticality and that applicant has not met the burden of showing that the instantly claimed ranges are critical to operation of preparing a solution that contains an ester of 5-ALA.

The Examiner states that Gierskcky teaches concentrations of the compounds of about 1 to 50%, the use of chelating agents such as deferoxamine, and methods of preparing and using ALA hexyl ester. The Examiner further states that all elements of the instant claims are described in Gierskcky, except the instantly claimed ranges of esters of ALA. According to the Examiner, in the absence of showing a criticality, it would have been *prima facie* obvious to optimize the concentration of Gierskcky's ALA-esters and their respective pH ranges to achieve a desirable clinical outcome.

The Examiner asserts that contrary to applicants' argument that no knowledge was generally available to one of ordinary skill in the art to arrive at the claimed concentrations of ALA-ester doses lower than 1%, articles cited in applicants' declaration, Lange et al., Peng et al. and Marti et al. that "show esterified aminolevulinic acid derivatives induce porphyrin fluorescence in skin" and that "such teachings were available to one of ordinary skill in the art because the artisans before filing of the instant application knew them." The Examiner concludes that optimization of a formulation to achieve such end-result would have been within the skill of the ordinary artisan and available at the time of the claimed invention.

The Examiner has deemed the Declaration under 37 C.F.R. 1.132 insufficient to overcome the rejection of claims 19-27 and 29-35 based on Gierskcky because it is not commensurate with the scope of the claims and does not provide adequate data comparing the

formulations of Gierskcky to establish unexpected results or criticality of lower ranges of ALA esters of the claimed invention. The Examiner states that the declaration explains methods of using esters of ALA at lower concentrations than conventionally employed in the art at the time of publication of the cited references and further, that such studies did not describe unexpected observations in formulating lower concentrations of esterified ALA. The Examiner asserts that the arguments are not commensurate with the scope of the claims because the instant claims are not directed to methods of using such compositions.

The Examiner also states that applicant has not provided any comparative data between esterified formulations of Gierskcky and the instant claims. The Examiner further asserts that Gierskcky even teaches esterified compositions at concentrations 1% or lower (citing Examples 1-5). Finally, the Examiner states that no data was submitted in the Declaration that the instantly claimed formulations provide unexpected results over Gierskcky or the concentrations of esterified ALA in the instant compositions are critical for preparing the claimed compositions.

Applicants respectfully traverse the rejection and maintain that the pending claims are not *prima facie* obvious over Gierskcky.

A. Gierskcky provides no suggestion or motivation to modify the reference to arrive at the claimed invention

Applicants note that no suggestion or motivation is provided by Gierskcky to modify the reference to arrive at the claimed invention.

Gierskcky states that generally, concentration ranges of 1 to 50% (w/w) are suitable and that for therapeutic applications, concentration ranges of 10 to 50% have been found to be suitable, *e.g.,* 15 to 30% w/w). One of skill in the art would not have been motivated to make pharmaceutical preparations comprising an ester of 5-aminolevulinic acid (E-ALA) ... which is dissolved in the solvent at a concentration of less than 1% by weight, as presently claimed, based on this disclosure. Accordingly, Gierskcky provides no suggestion to one of ordinary skill in the art to prepare the presently claimed pharmaceutical preparations comprising an ester of 5-aminolevulinic acid which is present in the preparation at a concentration of less than 1% by weight.

B. Gierskcky provides no reasonable expectation of success of the presently claimed invention

1. At the priority date of the subject application, in April 1998, the concentrations of ALA-esters studied were about two orders of magnitude higher than presently claimed.

Applicants respectfully again direct the Examiner's attention to the Declaration under 37 C.F.R. §1.132 of Georges Wagnières, Ph.D., (hereinafter Dr. Wagnières), which was submitted on January 26, 2004 together with applicants' amendment. Dr. Wagnières points out in paragraph 14 that at the time of the priority date of the subject application, in April 1998, the concentrations of ALA-esters studied were ***about two orders of magnitude higher*** than the concentrations of the present invention. As an example of such studies, Dr. Wagnières cites Peng et al. "Build-up of esterified aminolevulinic-acid-derivative-induced porphyrin fluorescence in normal mouse skin" J. Photochem. Photobiol B: 34 (1996):95-96 (which was attached thereto as Exhibit I), who administered methylester, ethylester and propylester of ALA in a cream in a concentration of **about 20% (w/w)**.

In comparison, the studies of Dr. Wagnières and colleagues administered a dose of approximately **0.2% (w/w)**, which is about two orders of magnitude lower than that used in Peng et al. Therefore, contrary to the Examiner's assertion, Peng et al. would not have provided a reasonable expectation that compositions comprising much less than 20% of esters of ALA, indeed less than 1%, would be successful for the same applications, *e.g.,* build-up of fluorescence after topical application of an ALA-ester.

Pharmaceutical preparations are manufactured for particular indications, *i.e.*, for specific uses. Based on the much higher concentrations of ALA-esters that were used at the time of the priority date of the subject invention, as discussed by Dr. Wagnières, one of ordinary skill in the art would **not have had any expectation that much lower concentrations of ALA-esters**, such as those presently claimed, would have been **therapeutically or diagnostically useful**. Further Gierskcky's teaching of suitable concentration ranges of 1 to 50% (w/w) would not have provided a basis for a reasonable expectation of success of compositions comprising an ALA-ester concentration of less than 1% by weight for use in either photochemotherapy or diagnosis.

2. Lange et al. and Marti et al. were published after the effective filing date of the present application.

The Examiner asserts that in addition to Peng et al., discussed above, Lange et al. and Marti et al. would have taught the one of skill in the art to arrive at the claimed concentrations of ALA-ester doses lower than 1%, because the artisans knew of the articles before filing of the instant application.

Applicants respectfully wish to point out that both Lange et al. and Marti et al. were published after the filing date of PCT International application number PCT/CH99/00163, of which the subject application is a U.S. national stage application under 35 U.S.C. §371.

Lange et al. was published in May 1999, *i.e.*, after the April 22, 1999 filing date of the PCT International application, which is the effective filing date of the subject application, and therefore, would not have been available to one of skill in the art before the filing date of the subject application. *See* Br. J. Cancer (1999) 80(1/2), 185-193, attached hereto at Tab 1. (Date Stamp of May 5, 1999 indicates the date of receipt by the Clinical Sciences Center of the University of Wisconsin - Madison)

Marti et al. was published in July 1999, *i.e.*, after the filing of the subject application, and therefore, would not have been available to one of skill in the art before the filing date of the subject application. *See* J. Urology (August 1999) 162(2), 546-552, attached hereto at Tab 2. (Date Stamp of July 15, 1999 indicates the date of receipt by the Clinical Sciences Center of the University of Wisconsin - Madison) Accordingly, contrary to the Examiner's assertion, these articles would not have been available to one of skill in the art at the time the PCT application was filed.

Therefore, Gierskcky would not have provided one of ordinary skill in the art with a reasonable expectation of clinical success of a pharmaceutical preparation comprising an ester of 5-aminolevulinic acid (E-ALA) ... which is dissolved in the solvent at a concentration of less than 1% by weight, as presently claimed.

3. Unexpected Results

At the priority date of the subject application, one of ordinary skill in the art would have considered "clinical success" of ALA-esters as the production of levels of PpIX upon administration of the low dose ALA-esters to the same extent as the levels of PpIX achieved with the lowest doses of ALA-esters studied at the time of the present invention, *e.g.*, the higher doses of 20% (w/w) of ALA-esters, as described by Peng et al.

In fact, as described by Dr. Wagnières, it was unexpected that the **lower doses** of ALA-esters would produce **higher** levels of PpIX than the known lowest doses of ALA-esters studied at the time of the present invention.

Applicants respectfully direct the Examiner's attention to Exhibit E, entitled "Dose-response of Different ALA-esters on the Pig Bladder Model," which was attached to the Declaration Under 37 C.F.R. § 1.132 of Dr. Wagnières. The graph of ALA-hexylester clearly shows that ALA-hexylester produces PpIX fluorescence beginning at a concentration of about 1 mM, *i.e.,* **0.025% w/w**, reaches a maximum at about 4 mM, which is **0.1% w/w** (as shown in Wagnières Exhibit F, Table 1), continues to produce fluorescence until a decline at about 10 mM (**0.25% w/w**), and produces no fluorescence at a concentration of 100 mM (**2.5% w/w**). **Applicants attach a copy of modified Exhibits C, E and H, in which the concentrations are shown as percentages, rather than in mM, as originally submitted with Dr. Wagnières Declaration.** *See* Tabs 3-5, respectively. The previously provided results, now shown as percentages, provide a basis for comparison with Gierskcky's formulations, which are disclosed as concentration ranges in percent (w/w).

Exhibit E also illustrates that ALA-octylester begins to produce fluorescence at about 0.5 mM, *i.e.,* **0.013 % w/w**, reaches a maximum at about 2 mM, *i.e.,* **0.056% w/w**, and precipitates at about 4 mM, concentration above which it does not produce any fluorescence. **Applicants note that the formation of precipitation at the aforementioned low doses does not permit testing of ALA-octylester at higher doses.**

The graph also shows that ALA-butyl produce fluorescence beginning just under 1mM, which is **0.0223 % w/w**. ALA-butyl ester decreases production of fluorescence at a concentration of over 20 mM, *i.e.,* **0.446% w/w**. These results demonstrate that higher concentration of these ALA-esters, such as those disclosed by Gierskcky, produce decreasing amounts of fluorescence.

Marti et al., which was attached as Exhibit D to Dr. Wagnières Declaration, state that "the reduction of fluorescence at high concentrations may indicate significant cytotoxicity of ALA esters which resulted in peeling of mucosa … or even immediate cell lysis (as with H- and B-ALA at 180 mM) (not shown)." *See* Marti et al. page 549, Col. 2, ¶2. Applicants note that 180 mM of ALA-hexylester and ALA-butylester is 4.5% w/w and 4.01% w/w, respectively, which correspond to the lower values of Gierskcky's concentration range. Therefore, these results demonstrate that concentrations lower than Gierskcky's lower limit of 1% w/w unexpectedly produce higher levels of fluorescence and that, as the concentration approaches the higher concentration percentages disclosed by Gierskcky, *i.e.,* well over the

1% w/w and up to the 50% w/w, cell toxicity results, thus reducing the level of PPIX fluorescence. Therefore, applicants have clearly demonstrated unexpected results over the concentrations disclosed in Gierskcky.

Applicants wish to add parenthetically that one of skill in the art would not have expected such results based on the fluorescence produced by ALA, since a much higher concentration of ALA produces the fluorescence needed for diagnosis or therapy. Exhibit E shows that the low doses of all of the ALA-esters tested produce much higher levels of fluorescence, *i.e.,* about two-thirds higher than does the high dose of ALA.

C. Gierskcky does not teach all of the claim limitations

Gierskcky does not teach all the claim limitations of the presently pending claims, *i.e.*, a pharmaceutical preparation comprising an ester of 5-aminolevulinic acid (E-ALA) for generating protoporphyrin IX (PpIX) which is present in the preparation at a concentration of less than 1 % by weight.

The Examiner has acknowledged this fact. (See October 6, 2004 Office Action, page 3, last ¶, penultimate sentence and July 25, 2003 Office Action "*Gierskcky fails to use concentrations of ALA-esters in amounts less than 1% and further specify the instant ranges of pH.*"

Therefore, the Examiner's contradictory assertion that "Gierskcky even teaches esterified compositions at concentrations 1% or lower" is wholly unsupported by Gierskcky. (*See* October 6, 2004 Office Action, page 5, ¶1, second sentence) Applicants respectfully note that Gierskcky's Examples 1-5 do not provide pharmaceutical compositions which comprise a 5-aminolevulinate dissolved in a solvent at a concentration of less than 1% by weight. Rather, these examples prepare, *i.e.*, synthesize, the methyl-, ethyl-, n-propyl, n-hexyl-, and n-heptyl 5-aminolevulinate esters by reacting 5 aminolevulinic acid hydrochloride in a solvent (methanol, ethanol, n-propanol, n-hexanol, and n-heptanol, respectively), removing the excess solvent, and drying the product. For instance, in Example 1, one gram of 5 amino-levulinic acid hydrochloride is added to 200 ml methanol and 1 drop of concentrated HCl. This solution is not suitable for administration, *i.e.,* it is not a pharmaceutical preparation, as presently claimed. This solution is also not the final product, *i.e.*, 5-aminolevulinate (an ALA ester). The esterification process takes place overnight and excess methanol is removed by distillation, followed by drying under vacuum. The *dried product is the 5-aminolevulinate (ALA ester)*, which may be used to prepare a pharmaceutical

composition. Therefore, Gierskcky does not teach a pharmaceutical preparation comprising an ester of 5-aminolevulinic acid (E-ALA) ... which is dissolved in the solvent at a concentration of less than 1% by weight, as presently claimed.

Since Gierskcky has not met the three criteria to establish a *prima facie* case of obviousness, the cited reference cannot render obvious the presently pending claims. Moreover, applicants have provided a showing of unexpected results over the concentrations disclosed by Gierskcky. Accordingly, Applicants respectfully request reconsideration and withdrawal of the rejection of presently pending claims 19-27 and 29-35 under 35 U.S.C. §103.

In view of the foregoing amendments and remarks, it is respectfully submitted that the present application is in condition for allowance, which action is earnestly solicited.

Respectfully submitted,

Dated: June 27, 2005

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Photodetection of early human bladder cancer based on the fluorescence of 5-aminolaevulinic acid hexylester-induced protoporphyrin IX: a pilot study

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Summary Exogenous administration of 5-aminolaevulinic acid (ALA) is becoming widely used to enhance the endogenous synthesis of protoporphyrin IX (PpIX) in photodynamic therapy (PDT) and fluorescence photodetection (PD). Recently, results have shown that the chemical modification of ALA into its more lipophilic esters circumvents limitations of ALA-induced PpIX like shallow penetration depth into deep tissue layers and inhomogeneous biodistribution and enhances the total PpIX formation. The present clinical pilot study assesses the feasibility and the advantages of a topical ALA ester-based fluorescence photodetection in the human bladder. In this preliminary study 5-aminolaevulinic acid hexylester (h-ALA) solutions, containing concentrations ranging from 4 to 16 mM, were applied intravesically to 25 patients. Effects of time and drug dose on the resulting PpIX fluorescence level were determined in vivo with an optical fibre-based spectrofluorometer. Neither local nor systemic side-effects were observed for the applied conditions. All conditions used yielded a preferential PpIX accumulation in the neoplastic tissue. Our clinical investigations indicate that with h-ALA a twofold increase of PpIX fluorescence intensity can be observed using 20-fold lower concentrations as compared to ALA.

Keywords: 5-aminolaevulinic acid; 5-aminolaevulinic acid hexylester; photodynamic therapy; fluorescence; protoporphyrin IX; human bladder cancer

Fluorescence photodetection (PD) and photodynamic therapy (PDT) are techniques currently under clinical assessment for both visualization and local destruction of malignant tumours and premalignant lesions. One drawback of these methods found with some photosensitizers is a more or less long-term cutaneous photosensitivity (Wagnières et al, 1998; Dougherty et al, 1990). A more recent strategy for administering photosensitizers involves the application of 5-aminolaevulinic acid (ALA) in order to stimulate the formation of protoporphyrin IX (PpIX) in situ. The exogenous ALA bypasses the negative feedback control from haem to ALA synthase that catalyses the condensation of glycine and succinyl–coenzyme A (CoA). Given in excess, exogenous ALA thus can result in a temporary accumulation of PpIX, in particular, in cells with higher metabolic turnover. Since PPIX has fairly good photosensitizing properties (Cox et al, 1982; Kennedy et al, 1990) proposed ALA as a possible photodynamic agent. Following this pioneering work, this treatment modality has been widely studied for various cancers (Kennedy et al, 1992; Peng et al, 1992; Svanberg et al, 1994).

As well as for the PDT of malignant or premalignant lesions, ALA-induced PpIX is now being used for the detection of such lesions. This technique has been shown to work, among other applications, in urology, where easy instillation in the bladder, combined with the fact that this organ is readily accessible endoscopically, makes it an ideal object. Alongside classical techniques such as cytology or white light examination, fluorescence PD by ALA-induced PpIX provides some advantages (Leveckis et al, 1994; Kriegmair et al, 1996; Jichlinski et al, 1997). This inspection modality allows an exact mapping which pinpoints, with a high level of sensitivity and specificity, the locations of carcinoma in situ (CIS) as well as early stages of cancer-like dysplasias, which are normally difficult to recognize under white light examination.

However, when using topically instilled ALA for the PDT of CIS and precancerous lesions, this modality appears to be limited by the amount of ALA that enter the target cells or by the tissue penetration and the distribution of the resulting PpIX in the targeted tissue. Almost all of these possible disadvantages accompanying the use of ALA can be ascribed to the physical–chemical properties of the molecule itself. Applied under physiological conditions, ALA is a zwitterion (Novo et al, 1996). Because the lipid bilayer of biological membranes is relatively impermeable to charged molecules, the cellular uptake of ALA is shallow. Consequently, in order to increase the transport across cellular membranes, fairly high drug doses and increased administration times have to be used. This deficiency results in a low penetration depth (Warloe et al, 1992; Loh et al, 1993; Peng et al, 1995) and an ALA-induced PpIX distribution, which is not optimized for the PDT of the deep layers of nodular lesions in the urothelium (Iinuma et al, 1995; Chang et al, 1996) after topical ALA application.

Systematic studies have shown that the modification of a drug to an ester, an amide or a urethane by the addition of a long-chain hydrocarbon improves penetration through biological barriers (Bridges et al, 1979; Jain, 1987*a*, 1987*b*). After penetration into the cell, the ester derivative can then, for example, be hydrolysed back to the free ALA by non-specific esterases. Recently,

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promising results were obtained with different alkylesters of ALA in vivo and in vitro (Kloek et al, 1996; Peng et al, 1996; Gaullier et al, 1997; Marti et al, 1998). These groups demonstrated that the application of esterified ALA derivatives results in an up to 25-fold increase in PpIX fluorescence levels as compared to ALA.

This report covers initial clinical investigations with 5-aminolaevulinic acid hexylester hydrochloride (h-ALA)-induced fluorescence PD in the human bladder. Following our preclinical studies (Marti et al, 1999), we selected h-ALA from the multitude of possible ALA-alkylesters because it represents a good compromise between water–urine solubility and sufficient PpIX formation capacity at low doses. Furthermore, h-ALA has been shown to lead to a homogenous distribution of PpIX-related fluorescence over the entire urothelium in our pig bladder model (Marti et al, 1998). In addition, it can be synthesized simply from ALA and hexanol (Kloek et al, 1997). The goal of this clinical pilot study was to test h-ALA as a potential candidate for improving both the PD and PDT in the urinary bladder. Therefore, topical application of h-ALA should result in higher PpIX formation than is the case with the same amount of ALA. It should enable shorter times between instillation and examination and lower drug concentrations while retaining the outstanding selectivity of ALA. This work presents a preliminary optimization of h-ALA-induced PpIX in respect to the resulting fluorescence intensities. Both the influence of the concentration and instillation time of h-ALA solutions on the total amount of PpIX were determined in vivo by the use of an optical fibre-based spectrofluorometer.

MATERIALS AND METHODS

Patients

Twenty-five patients (seven women and 18 men, four cases of ordinary ALA and 21 cases of h-ALA) have been involved in this first study conducted since August 1997. The mean age was 70 years, covering an age range of between 44 and 85. Local ethical committee approval was granted for this study, and written consent was obtained in each case.

Preparation and administration of ALA and h-ALA

ALA (99%) was purchased from Merck (Darmstadt, Germany). Other chemicals (thionyl chloride 99% and 1-hexanol 99.9%) used for the synthesis of h-ALA were ordered from Fluka Chemie AG (Buchs, Switzerland) and were used without further purification.

The synthesis described here is a slight modification of the methods reported recently (Takeya, 1992; Kloek et al, 1996). In brief, 3.5 ml of thionyl chloride were added drop by drop under stirring to an excess (~ 10 ml) of 1-hexanol cooled on ice in an

Table 1 Experimental instillation conditions used in the first clinical trials with h-ALA and normalized fluorescence levels on papillary tumours (pTa G2) obtained by normalization to reference cuvette

Patient no.	Concentration (mM)	Instillation time (h)	Resting time (h)	Fluorescence signal (r.u.)
1	4	2	–	16.2
2	4	2	–	11.2
3	4	4	–	34.5
4	4	4	–	20.5
5[b]	8	2	–	22.1
				38.4
6	8	2	–	36.2
7	8	2	–	46.7
8	8	2	–	–
9[b]	8	2	2	151.1
				102.0
10	8	2	2	115.8
11	8	2	2	147.5
12	8	4	–	66.4
13	8	4	–	72.6
14	8	4	–	63.4
15	8	4	–	73.8
16	8	4	–	94.4
17	8	4	–	77.1
18	8	4	2	102.7
19	8	4	2	95.0
20	16	2	–	15.8
21	16	2	–	16.7
22	180[a]	4	2	54.0
23	180[a]	4	2	43.6
24	180[a]	4	2	46.2
25	180[a]	4	2	45.3

[a]Instillation of the 180 mM solution of ALA.[b]Patient with two papillary tumours.

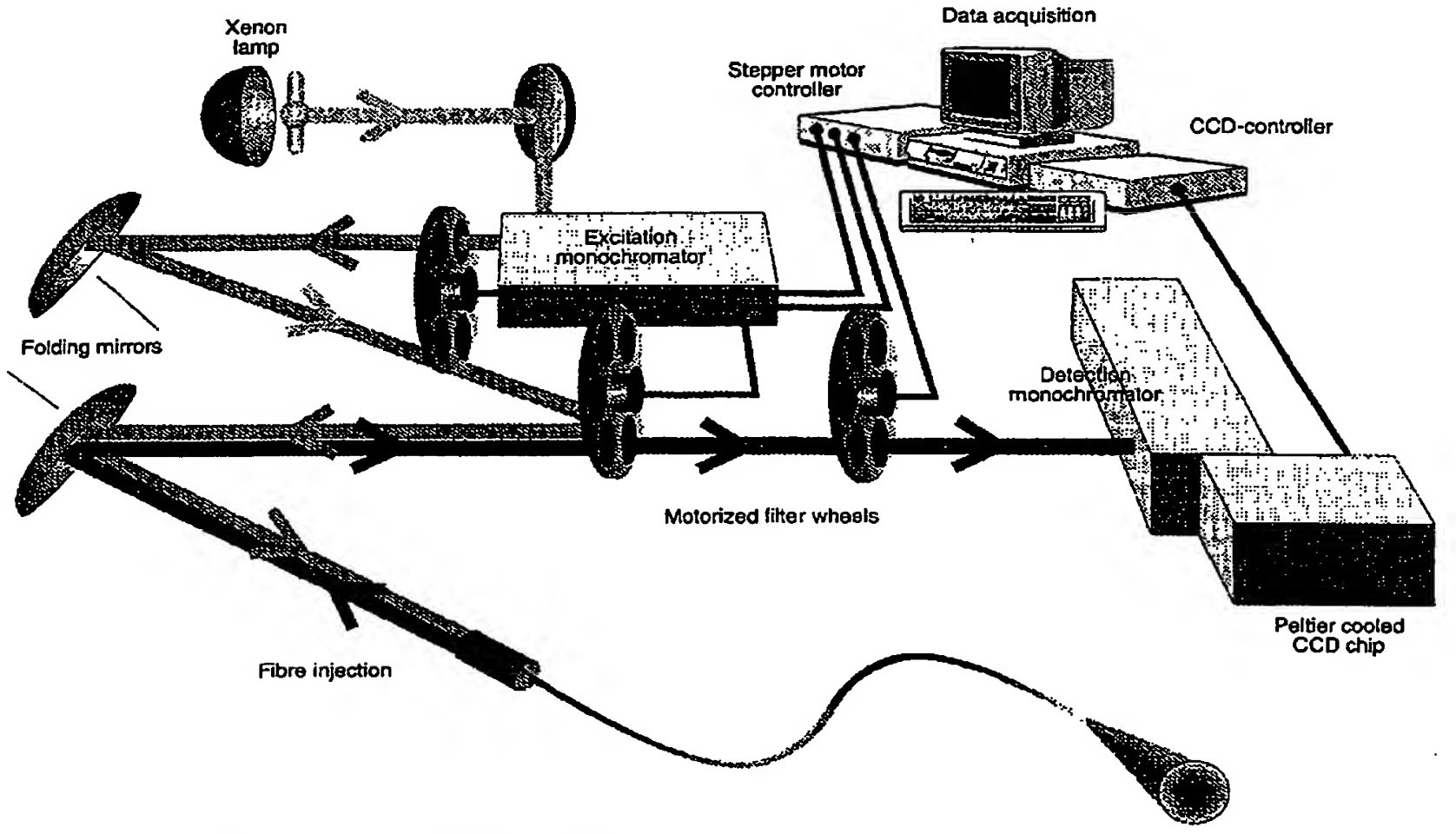

Figure 1 Schematic view of the optical fibre-based spectrofluorometer

argon atmosphere. The solution was stirred for a further 30 min to bring the reaction to completion; after warming up to room temperature, 2.5 g of ALA (M_r = 167.6 g mol^{-1}) were added to the solution. The suspension was then stirred overnight at room temperature under argon. The final phase of the reaction was controlled on-line by thin layer chromatography (TLC) (TLC foils; Schleicher & Schuell, Merck, Darmstadt, Germany) in CH_2Cl_2/MeOH (9:1) stained by $KMnO_4$ (Rf = 0.6). Once the reaction was complete, the solvent and hexylchloride were removed under reduced pressure (~ 0.5 torr). The viscous residue was dissolved in warm methanol. Then a small amount of methanol was evaporated until the first crystals of the reaction product appeared. A small quantity of diethylether was added and h-ALA was allowed to crystallize on ice. This dissolving and recrystallizing procedure was then subsequently repeated until only one spot was recognized on the TLC, yielding 80–90% of h-ALA (M_r = 251.8 g mol^{-1}) as a white powder. The product was characterized by proton nuclear magnetic resonance (^{1}H-NMR) with a 400 MHz (Bruker, Germany) spectrometer and identified as 5-aminolaevulinic acid hexylester hydrochloride. The purity (> 95%) was further verified by high performance liquid chromatography (HPLC) with UV/VIS detection at 270 and 350 nm respectively (data not shown). No other products were observed.

The ALA solutions were administered in accordance with standard protocol used in Lausanne's CHUV Hospital (Jichlinski et al, 1997). In brief, 1500 mg of ALA were dissolved in 38 ml of sterile water. Five millilitres of phosphate-buffered saline (PBS) were added and the pH was adjusted with a further 7 ml of aqueous sodium hydroxide (1N) to a value of pH 5.3. This solution with a concentration of 180 mM of ALA was sterilized by filtration through a Millipore filter (Millipore, Millex GS 0.22 µ) and stored at –18°C 1 day before measurements were conducted. The solution was instilled into patients' bladders using a 16 French Foley catheter 6 h prior to photodetection. Patients were asked to retain the solution for 4 h. Their bladders were evacuated 2 h prior to treatment.

Depending on the prodrug concentration to be applied, 50–200 mg (i.e. 4–16 mM) of crystalline h-ALA were dissolved in 35 ml of water. Then 13 ml of PBS were added to the aqueous solution and adjusted with 0.1 N hydrochloric acid to give the same pH value of 5.3. The solutions were instilled as described above. Table 1 summarizes the different conditions under which ALA and h-ALA were applied. All patients treated with ALA (four cases) and some instilled with h-ALA (five cases) had a supplementary resting time of 2 h after being exposed to the drug solution.

Procedure

Bladder inspection under white light illumination

Prior to further treatment or measurement, the actual status of the bladder was documented under white light illumination. The frame accumulation colour CCD camera (Storz, Tuttlingen, Germany), connected to a video recorder (JVC, Japan) and an RGB monitor (Sony, Japan) was plugged directly into the ocular of a 23.5 French cystoscope (Storz PDD, Tuttlingen, Germany) to record the standard endoscopic colour image.

Fluorescence spectroscopy

Fluorescence emission spectra were recorded with an optical fibre-based spectrofluorometer based on a Peltier-cooled CCD coupled to a spectrograph (Cromex 250, SI Instruments, Germany). The experimental setup is shown in Figure 1. Arranged on a trolley, the whole setup can be easily transported. Excitation light (λ_{ex} = 405 nm) from a 75 W high-pressure Xenon lamp (UXL-75 XE, Ushio Inc., Japan) was spectrally resolved by a quarter meter monochromator (Chromex 250, SI Instruments, Germany) with a bandwidth of 5 nm and an excitation filter, SCHOTT BG3 (Schott AG, Mainz, Germany), mounted on a filter wheel. A stepper motor (SMC 100, Princeton Instruments Inc., USA) controlled this excitation filter wheel, which was equipped with different low-pass filters installed to purify the excitation light. Fully reflective mirrors and a dichroic mirror (Reynard DC 450; Reynard, USA), mounted on a second filter wheel, were used to feed the light into a 600 µm core silicone-clad silica fibre with perpendicular polished end-faces. Excitation energy measured at the distal end of the fibre tip was determined with a calibrated power-meter (Optical Power Meter 840, Newport, USA). Fluorescence emitted by any sample was collected with the same fibre and separated from the excitation light by the dichroic optics described above. A long-pass filter (Reynard FG 455) mounted on a third filter wheel made further spectral separation, virtually eliminating all reflected excitation light prior to acquisition. This filter setup allows the acquisition of fluorescence emission spectra between 450 and 900 nm. Detection based on this combination enables fast data acquisition combined with a low level of noise. The whole setup and data acquisition was controlled by a 486 personal computer using CSMA software (SI Instruments GmbH, Germany).

An aqueous solution of Rhodamine B ($c = 1 \times 10^{-6}$ mol l^{-1}) in a 10 mm quartz cuvette was used as a reference. Emission spectra of the reference were recorded before and after each measurement. All measurements were normalized to the peak value of the reference to give comparable results corrected for day-to-day fluctuations of the excitation light energy or detection pathway alignment.

After inspection of the bladder under white light, the distal end of the fibre was introduced via the biopsy channel of the cystoscope. A background measurement was performed in the centre of the bladder to allow the correction of the spectra for parasitic light and fluorescence generated by the fibre itself. Then the physician brought the distal end of the fibre directly into contact with the bladder wall.

Bladder inspection under violet light illumination

After measurement of the fluorescence spectra (see below) of healthy, cancerous and suspicious areas in the bladder, the camera was equipped with a long-pass filter ($\lambda > 520$ nm; Wratten filter No. 12, Kodak, Rochester, USA), positioned between the ocular of the cystoscope and the CCD-Chip. A footswitch allows the physician to place a bandpass filter (380–450 nm) in front of the 300 W Xenon arc lamp (Storz, Tuttlingen, Germany) to give about 150 mW of violet light at the end of the cystoscope. Excitation with violet light generated a visible pale-green autofluorescence of the healthy mucosa. As a result of the absorption of autofluorescence, the blood vessels of the lamina propria appear somewhat darker. Filtration of the light below 520 nm allows these sites to be distinguishable from zones containing high PpIX concentrations, appearing in a clear, bright, fluorescing red. To improve the fluorescence images, the camera was switched into frame accumulation mode for enhanced sensitivity. The integration times ranged from one-eighth to one-half of a second, depending on observation distance.

Biopsy sampling and pathology

Prior to transurethral resection of the bladder wall (TURB), a total number of 109 biopsies from fluorescent and non-fluorescent areas (average 5.2 per patient; guided by light-induced fluorescence after excitation at 405 nm) were taken from the patients treated with h-ALA solutions. Macroscopic fluorescence findings and locations were documented for each biopsy. All samples were sent for histopathological examination. The urothelial carcinomas were graded and staged according to the World Health Organization (WHO) 1973 classification (Mostofio et al, 1973) and the UICC/AJC 1992 system (Hermanek and Subin 1992) respectively. Flat intra-epithelial neoplastic lesions were graded according to the criteria of Nagy et al (1982) and classified as grade 1 (mild dysplasia), grade 2 (moderate dyslasia), grade 3 (marked dysplasia) and carcinoma in situ.

RESULTS

Macroscopic findings

All aqueous solutions of h-ALA stayed clear and colourless until use. Neither systemic nor local reactions following the examination with both h-ALA and ALA were observed under the conditions used in this study. Even the highest drug dose administered (16 mM) of h-ALA was well tolerated. h-ALA-induced synthesis of PpIX was observed in each patient. All papillary and planar tumours, also visible under white light cystoscopy, showed bright red fluorescence. This red fluorescence was found to demarcate the outline of the urothelial lesions with high precision. Using the violet light of the filtered Xenon arc lamp, it was possible to perform both fluorescence-guided biopsies as well as accurate resections of targeted tissues. Qualitatively, all conditions tested resulted in a clearly visible contrast between healthy and diseased sites of the bladder wall.

Table 2 Correlation between histopathological finding and fluorescence diagnosis following h-ALA instillation

Histopathological findings	Total number of biopsies	Fluorescence positive	Fluorescence negative
Healthy mucosa	28	5	23
Metaplasia	1	1	–
Hyperplasia	3	3	–
Dysplasia G1	12	10	2
Dysplasia G2	5	3	2
Dysplasia G3	2	2	–
CIS	11	9	2
pTa G1	8	8	–
pTa G2	14	14	–
pTa G3	19	19	–
pT1 G2–G3	4	4	–
pT2a	2	2	–
Total	109	80	29

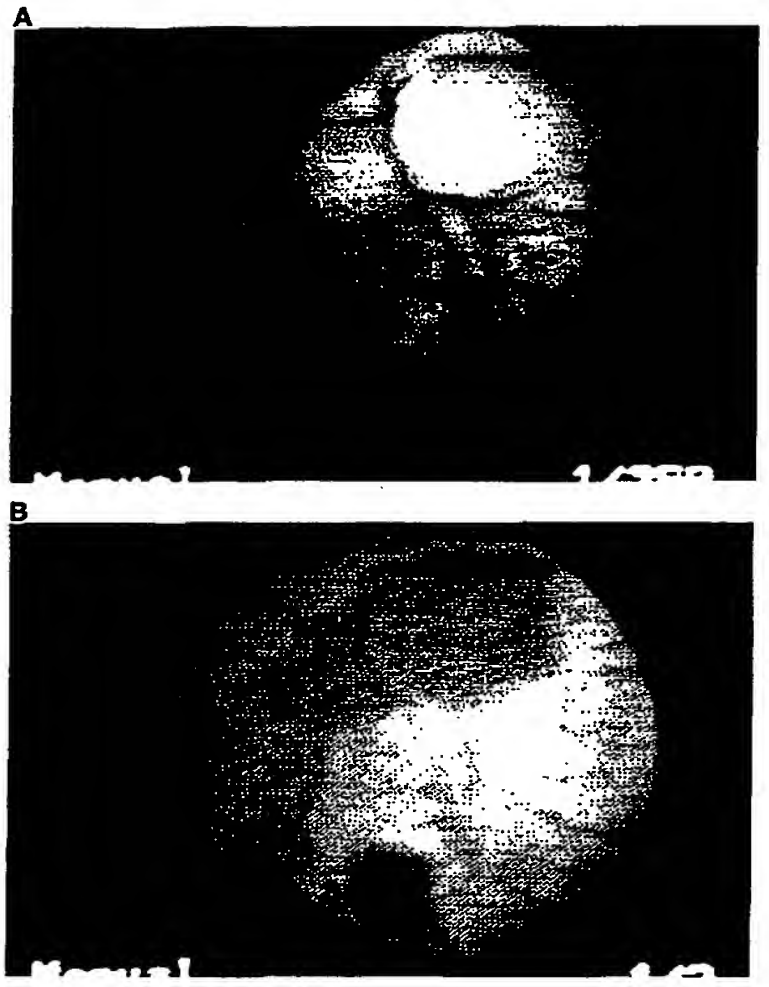

Figure 2 Endoscopic view of a flat papillary tumour (pTa G2) after 2 h of h-ALA exposure (for description see text) under (A) white light, (B) violet light examination

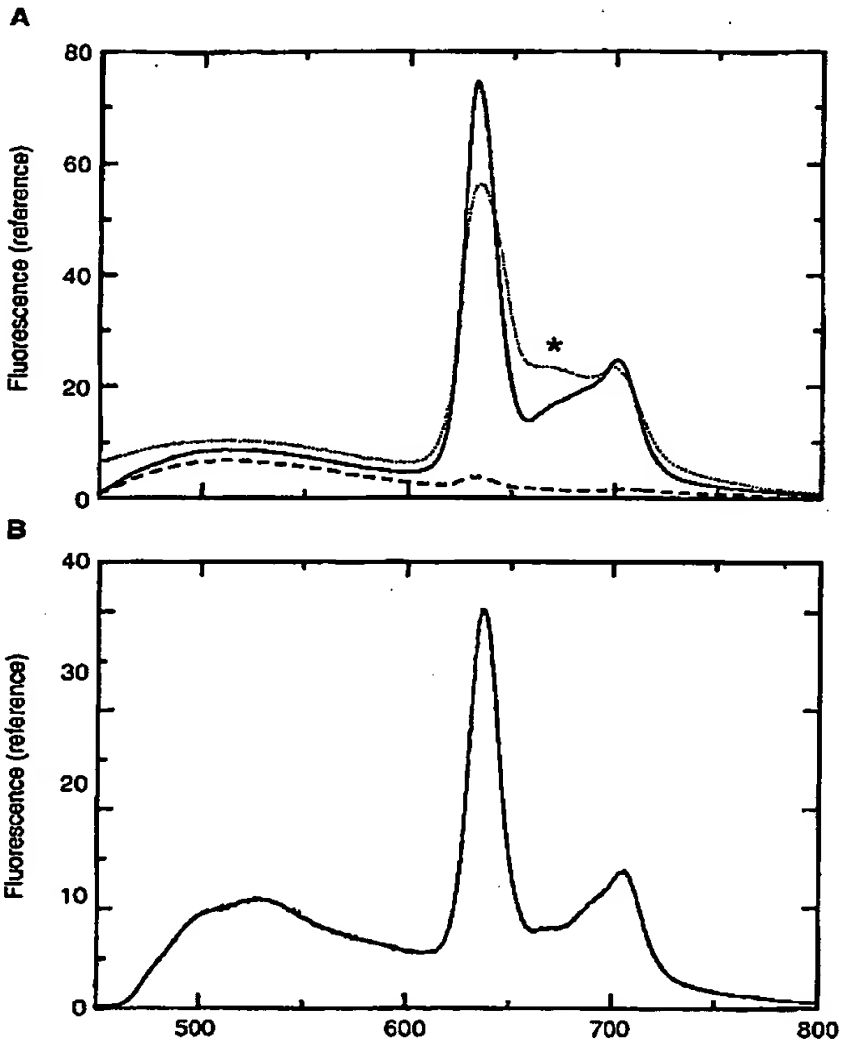

Figure 3 (A) Fluorescence spectra of h-ALA-induced PpIX (λ_{ex} = 405 nm) in normal mucosa (– – – –) and a papillary tumour (pTa G2, ——) after 4 h of instillation with 8 mM h-ALA (. . . .): a fluorescence peak (*) at 670 nm becomes visible due to photobleaching of PpIX. (B) Fluorescence spectra of ALA-induced PpIX (λ_{ex} = 405 nm) in a papillary tumour (pTa G2, ——) after 6 h of installation with 180 mM ALA)

Figure 2 demonstrates the advantageous use of h-ALA- induced PpIX for the fluorescence diagnosis of human bladder cancer. The two pictures show a sequence of a white light (Figure 2A) and a violet light (Figure 2B) examination after instillation of 8 mM of h-ALA over a period of 2 h (patient no. 10). White light illumination shows two papillary tumours (pTa G2) situated below the air bubble of the bladder under investigation. Fluorescence PD of the same area (Figure 2B) indicates a further lesion [flat papillary tumour (pTa G2)] which is barely detectable under white light.

Fluorescence findings and histopathological diagnosis

A total of 109 biopsies were taken under light-induced fluorescence from patients after instillation with h-ALA solutions. The correlation between the fluorescence findings and the histopathological analysis is summarized in Table 2. Thirty-two tissue samples were excised from healthy areas of the bladders investigated, containing eight samples, which were considered to be fluorescent. Histopathological diagnosis of the latter samples indicates the reasons for these 'false positive' responses. All these specimens showed tissular structures known for a higher cellular turnover, e.g. metaplasias, hyperplasias, chronic inflammation, or scar formation. In total, only six of the 77 biopsies taken from malignant and premalignant sites were not fluorescing. Three of these 'false negative' responses can be explained by nonoptimized conditions with regard to the concentrations of h-ALA applied (two moderate dysplasias; patient no. 4) as well as non-optimal incubation times (CIS; patient no. 1). A further CIS was missed (patient no. 13) probably due to an unusually long period of white light illumination preceding photodetection, resulting in the photobleaching of PpIX. Without exception, histopathologically-staged pta G1 or higher samples were found by fluorescence photodetection.

Fluorescence spectroscopy

Fluorescence emission spectra were measured on a total number of 24 patients (20 h-ALA, four ALA) (Table 1). ALA- and h-ALA-induced porphyrins were excited at 405 nm on both healthy and malignant areas in the human bladder. The emission from the urothelial surface was spectrally resolved between 450 and 800 nm. A comparison of the emission spectra after ALA and h-ALA exposure is plotted in Figure 3. The total fluorescence intensity is normalized to the reference. As shown in this Figure, the characteristic emission bands of PpIX at λ = 635 nm and λ = 708 nm after excitation in the Soret Band are clearly visible. According to the spectral shape, the fluorescence is attributed to PpIX. In none of the spectra recorded in vivo, an indication of porphyrins other than PpIX could be found. Depending on the duration of white and violet light examinations before fluorescence

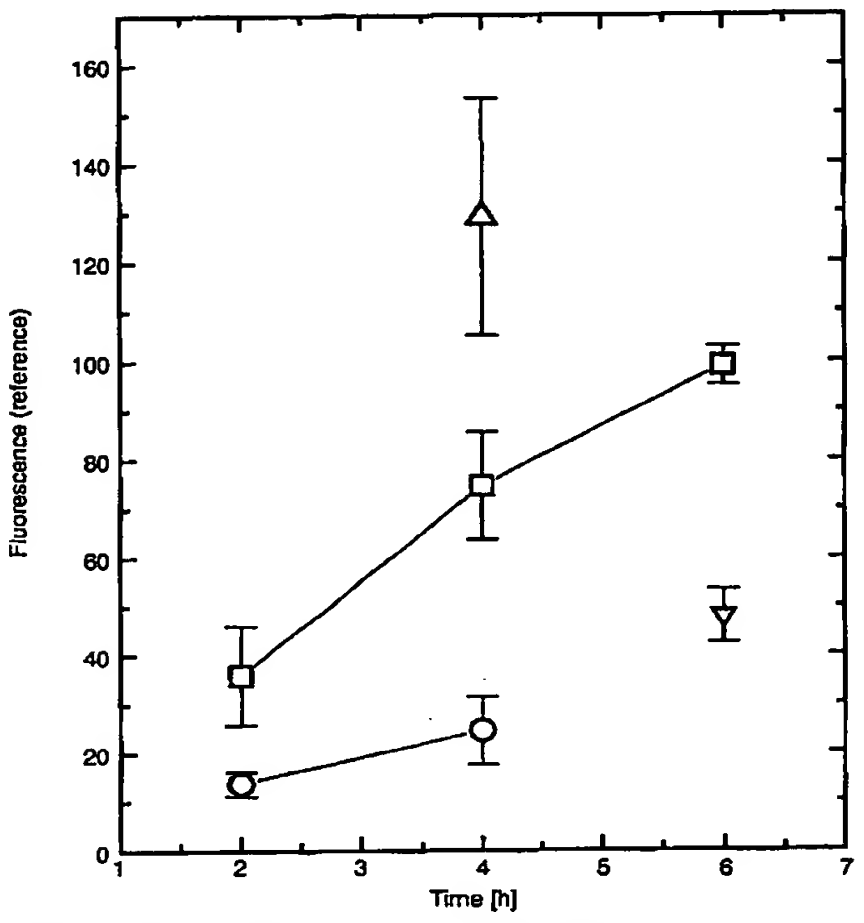

Figure 4 Effect of instillation time on the relative PpIX fluorescence intensity at 636 nm in papillary tumours (pTa G2) (□: 8 mM h-ALA; ○: 4 mM h-ALA; △: 8 mM h-ALA (2 h of instillation + 2 h of resting time); ▽: 180 mM ALA)

measurements, a peak, attributed to a PpIX photobleaching product, of around 665–675 nm appeared (dotted line in Figure 3A). The appearance of this supplementary fluorescence peak results in a line broadening because of overlapping fluorescence emission peaks and may suggest some degree of heterogeneity. In addition to the fluorescence emission spectra recorded on a papillary tumour pTa G2 after 4 h of h-ALA exposure, the corresponding spectra obtained on a healthy area were plotted (dashed line in Figure 3A). From these spectra, it can be seen that the healthy mucosa's autofluorescence of around 513 nm exceeds the two typical fluorescence peaks of PpIX at 636 nm and 708 nm. All samples taken from sites with these fluorescence characteristics were confirmed as healthy after histopathological examination. Evaluation of all fluorescence data available reveals that papillary tumour had the highest emission intensities. Premalignant lesions, such as dysplasias and carcinoma in situ, generally showed lower PpIX fluorescence intensities compared to malignant lesions. However, no direct relationship has been discovered between histopathological grading and relative fluorescence values.

Effect of exposure time and concentration

Three different solutions containing 50 mg (4 mM, four patients), 100 mg (8 mM, 15 patients) and 200 mg (16 mM, two patients) of h-ALA in 50 ml of the solvent were instilled in human bladders between 2 and 6 h prior to the fluorescence measurements (Table 1). Increased red fluorescence due to enhanced PpIX formation in pre-malignant and malignant lesions compared to the surrounding healthy sites was observed at all applied conditions. In order to quantify the resulting PpIX fluorescence, emission spectra were collected from different sites of the treated bladders. The fluorescence intensities of papillary tumours pTa graded G2 or G3 at 636 nm were chosen as standard in order to determine the influence of the different treatment conditions applied. This selection is based on the presence of this type of lesion in each bladder examined. Table 1 summarizes the influence of the different conditions on the relative fluorescence intensities of the PpIX emission band at 636 nm.

Analysis of the data available from patients exposed for 2 h (patient nos 1, 2, 5, 6, 7, 20 and 21) to different h-ALA concentrations indicates a strong concentration dependent on the PpIX fluorescence. It appears that, within 2 h, a solution of 8 mM h-ALA generates the highest fluorescence levels as compared to 4 mM and 16 mM of h-ALA. In Figure 4, the time course of the relative fluorescence intensity is plotted for h-ALA concentrations of 4 mM and 8 mM. An increase of fluorescence intensity with instillation time was observed in the two solutions. In addition, Figure 4 shows that, taking both the total fluorescence and the slope of the graphs into consideration, an instillation of 8 mM h-ALA solution is more efficient than that of a 4 mM solution.

In the course of our preliminary clinical study, a total of four patients (nos 8–11) were instilled under slightly different conditions. The patients' bladders were exposed to the solutions for 2 h. Following this exposure time, the bladders were emptied and the patients were allowed a supplementary resting time of 2 h. From Figure 4, it is clear that following this '(2+2)-concept' significantly enhanced fluorescence levels can be obtained compared to permanent exposure to the drug for 4 h.

The comparison of the relative fluorescence intensities of an 8 mM h-ALA solution and a 180 mM solution of ALA under similar conditions (4 h of instillation, 2 h of supplementary resting time) clearly demonstrates the advantage of using h-ALA. A treatment under these conditions with a topical 8 mM h-ALA solution resulted in a twofold increase of the fluorescence signal as compared to topical 180 mM ALA. After only 4 h of h-ALA exposure (8 mM), the relative fluorescence intensity already exceeded that induced by ALA (180 mM) 6 h after instillation.

DISCUSSION

Bladder cancer is a fairly common disease, appearing between the ages of 50 and 70 (Richie et al, 1989; Levi, 1993). This cancer, characterized by a high incidence (Levi, 1993), can appear in many distinct morphological forms, single or multiple, visible such as papillary or invisible such as 'flat' atypical lesions, mainly represented by low- or high-grade dysplasia or CIS. Bladder tumour multiplicity and the presence of these different forms of atypia are indicators of poor disease prognosis. Recognition of all visible or invisible lesions is therefore a prerequisite for any kind of treatment, with the aim of reducing the risk of progression or the rate of recurrence.

Although topical application of ALA has proved to be a helpful and reliable tool in fluorescence photodetection of invisible lesions in human bladder disease (Kriegmair et al, 1994; Jichlinski et al, 1996), some problems remain due to ALA's poor bioavailability. A small hydrophilic amino acid like ALA does not penetrate into all tissue compartments with great ease. Hence its concentration in tissue may remain relatively low and its distribution somewhat heterogeneous. Consequently, high drug doses over long instillation periods have to be used.

Three different concepts have been proposed to enhance the ALA-induced PpIX formation in deeper layers of the target tissue. Two of them are based on the use of chemicals, given along with ALA, in order to enhance both its penetration into deeper tissue layers and/or the total PpIX accumulation. This transepithelial penetration enhancement can be achieved either by prior dimethyl sulphoxide exposure of the targeted area (Peng et al, 1995) or by encapsulation of ALA into liposomes (Fukuda et al, 1992). The second approach uses agents interfering directly with the biosynthetic pathway of haem. Tetrapyrrol modulators, such as 1,10-phenanthroline (Rebeiz et al, 1996) and allyl-isopropyl-acetamide (AIA) (Schoenfeld et al, 1994) stimulating the enzymatic activity associated with PpIX formation. Iron chelators (e.g. ethylenediaminotetraacetic acid (EDTA) (Hanania and Malik, 1992; Orenstein et al, 1995; Warloe et al, 1995), desferrioxamine (DFO) (Ortel et al, 1993) CP94 (Chang et al, 1997) have been shown to increase PpIX concentration by preventing the ferrochelatase-mediated insertion of iron into the tetrapyrrol ring. This study followed a third approach, based on the thesis that the transformation of the hydrophilic ALA into more lipophilic prodrugs will enhance drug uptake.

In view of the results obtained using esters of ALA in vitro (Kloek et al, 1996; Gaullier et al, 1997; Marti et al, 1999; Tyrrell et al, 1993) and in vivo (Kloek et al, 1996; Peng et al, 1996), it appeared reasonable to envisage developing such a substance for clinical tests in which superficial bladder carcinoma is detected by fluorescence and possibly even treated by PDT. From the variety of derivatives recently tested in our laboratory (Marti et al, 1999), we selected h-ALA as it represents a good compromise between water–urine solubility and lipophilicity. It also gave an excellent in-vitro dose drug response compared to ALA solutions. Furthermore, it can be synthesized with a fairly high yield and low cost. The goal of this first clinical study with h-ALA in urology was to evaluate the feasibility of fluorescence photodetection with this new agent and the advantages achieved by instillation of h-ALA as compared to ALA for use in the human bladder.

One criterion for the use of h-ALA as a potential candidate in replacing ALA, is the preservation of the outstanding selectivity of ALA-induced PpIX for malignant and pre-malignant tissues. Confirmed by histopathological examination, we have demonstrated that the fluorescence of PpIX in the urothelium induced by intravesically administered h-ALA correlated significantly with neoplastic lesions and was suitable for the detection of papillary tumours as well as for dysplasia and carcinoma in situ. The 7% rate of false negative responses found in the present study is comparable to the value given by Jichlinski et al in 1997 and slightly higher than that presented by the Munich group (Kriegmair et al, 1996). A total number of 28 biopsies were taken from areas proven to be benign. Only five of these samples revealed an enhanced red fluorescence under violet light irradiation, yielding a rate of falsely positive fluorescence findings of 17%. This result seems to be quite small compared to both the results of Kriegmair et al (1996) and Jichlinski et al (1997). But it may be explained by the small number of biopsies taken, or the fact that the fluorescence induced by the long-chain esters was found to be limited to the site of application (Peng et al, 1996), hence no supplementary PpIX build-up from systemic ALA uptake is observed.

Clinical fluorescence spectroscopy has been used for measuring the PpIX accumulation kinetics, indicating an increase of h-ALA-induced PpIX with time in the human bladder within 6 h. A quantitative comparison of the fluorescence intensities at 636 nm following similar instillation conditions with solutions of 180 mM of ALA or 8 mM of h-ALA, respectively, clearly shows the advantages of h-ALA-induced PpIX. The more than twofold increase of the fluorescence signal due to the use of h-ALA is in good agreement with the in vivo results of Kloek et al (1996).

The time course of the PpIX fluorescence intensity in neoplastic tissues shows that, following 8 mM h-ALA exposure for 2 or 4 h, synthesis of PpIX continues within almost 2 h after termination of the instillation. In this time range, the fluorescence intensity increases 400% (2 h of exposure, 2 h of resting time) and 25% (4 h of exposure, 2 h of resting time) respectively.

The significant increase of the fluorescence signal using the '(2+2)-concept' as compared to a permanent exposure to drug for 4 h, as well as the strong dependence on the instilled h-ALA concentration, can be explained by an interference of high ALA concentrations with the biosynthetic pathway of haem. This observation was confirmed by in vitro experiments made by Gaullier et al (1997) and Marti et al (1999) with several ALA esters including h-ALA. Whereas the transport of ALA across the lipid bilayer of cell membranes probably represents a bottleneck in the PpIX formation, the enhanced uptake of lipophilic h-ALA may saturate the intracellular PpIX biosynthesis. This saturation might cause a negative feedback to enzymatic activity. Furthermore, high intracellular ALA concentrations have been shown to be cytotoxic. A high cellular ALA content may induce the release of Ca^{2+} from mitochondria, mitochondria swelling and uncouple respiration (Hermes-Lima, 1995). It can also cause ferritin iron release (Berg et al, 1996) or mediate the formation of 8-hydroxy-2′-deoxyguanosine in DNA (Fraga et al, 1994).

The results presented in this study have shown that a 2 h instillation of h-ALA (8 mM) provides sufficient PpIX fluorescence for reliable photodetection of malignant and pre-malignant lesions. This reduction in instillation time to only 2 h significantly increases the patient's comfort. Moreover, this makes outpatient treatment feasible and helps to cut costs in view of the excessively increasing cost of hospitalization. Finally, the reduction of the drug dose will decrease drug cost and the potential risk of mild complications provoked by ALA, recently reported by Rick et al (1997).

While for reliable fluorescence photodetection a 2 h instillation of 8 mM h-ALA has been shown to give satisfactory results, other conditions must be fulfilled with respect to an efficient bladder cancer therapy by PDT. Among other factors, the two key parameters of high concentration of the photosensitizer and its homogeneous distribution in the target tissue play a major role for the effectiveness of PDT.

Fluorescence microscopic studies showed that, after topical application of ALA, the PpIX was restricted to the superficial layers of the bladder tumours (Steinbach et al, 1994). On the contrary, preliminary fluorescence microscopic studies on some biopsies, taken in this study (data not shown), as well as the in vitro studies of Marti et al (1999) demonstrated homogeneously distributed PpIX fluorescence over the entire urothelium after topical application of h-ALA solutions.

Considering the photobleaching of porphyrins during irradiation (Rotomski et al, 1996; Bezdetnaya et al, 1996; Moan et al, 1997), a threshold concentration of PpIX necessary for tissue destruction, and the high selectivity of h-ALA-induced PpIX, a small PpIX amount in healthy areas of the bladder, observed in this study will

probably not induce any damage in these regions. However, the twofold increase of PpIX fluorescence after 6 h in neoplastic tissues by using h-ALA may further enhance the PDT effect as compared to the use of ALA. The latter appeared insufficient as observed in recent studies (Kriegmair et al, 1996).

It can be concluded that the use of h-ALA is a promising way to improve the photodetection of neoplastic and pre-neoplastic lesions as compared to ALA. In future, h-ALA may replace the use of ALA for clinical intravesical instillation because it is easy to use, real time observation without major auxiliary devices is possible and it is relatively cheap. Finally, it looks more promising as a PDT agent than ALA itself.

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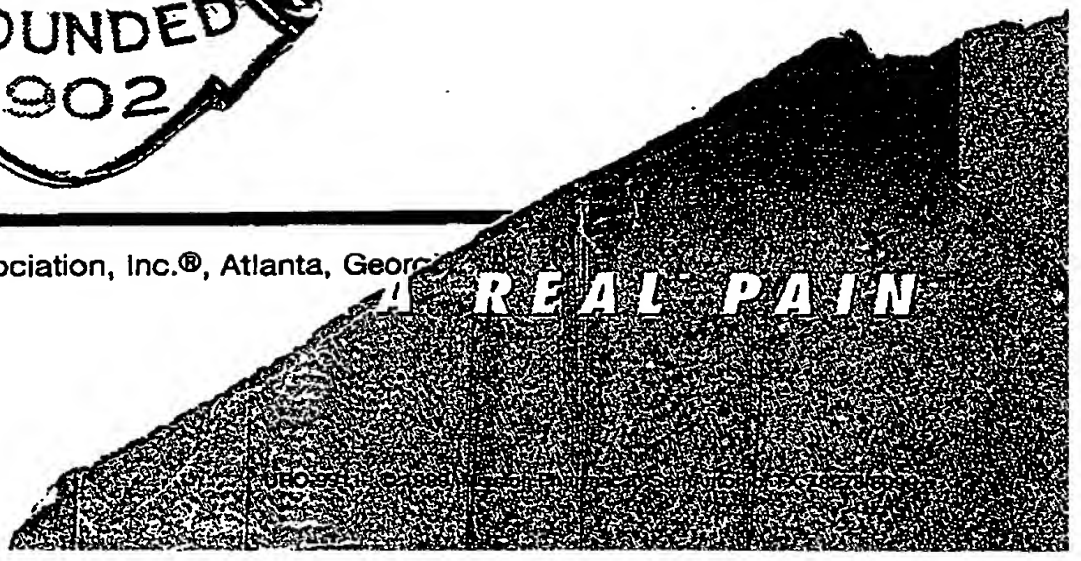

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OPTIMISATION OF THE FORMATION AND DISTRIBUTION OF PROTOPORPHYRIN IX IN THE UROTHELIUM: AN IN VITRO APPROACH

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ABSTRACT

Purpose: To optimize conditions for photodynamic detection (PDD) and photodynamic therapy (PDT) of bladder carcinoma, urothelial accumulation of protoporphyrin IX (PpIX) and conditions leading to cell photodestruction were studied.

Materials and Methods: Porcine and human bladder mucosae were superfused with derivatives of 5-aminolevulinic acid (ALA). PpIX accumulation and distribution across the mucosa was studied by microspectrofluorometry. Cell viability and structural integrity were assessed by using vital dyes and microscopy.

Results: ALA esters, especially hexyl-ALA, accelerated and regularized urothelial PpIX accumulation and allowed for necrosis upon illumination.

Conclusions: hexyl-ALA used at micromolar concentrations is the most efficient PpIX precursor for PDD and PDT.

KEY WORDS: aminolevulinic acid, photodynamic detection, photodynamic therapy, urinary bladder, cancer, in vitro

Urinary bladder tumors show an increasing incidence in man after the sixth decade. They consist mainly of superficial transitional carcinomas and are characterized by frequent recurrence and/or risk to progress toward invasive tumors.[1] This is linked to their frequent multifocal character and concomitant presence of high grade dysplasia (DYS) centers and/or carcinomas in situ (CIS).[2]

The treatment of superficial bladder tumors is based mainly on endoscopic resections combined with chemo- or immunotherapy by intravesical installation. While the use of BCG is likely to modify the recurrence profile of the illness, reduce the risk of progression and improve survival,[3] the resistance to BCG of certain tumoral bladders and decrease of vesical compliance resulting from repetitive treatments remain therapeutic problems.

As an alternative, photodynamic therapy (PDT) aims at destroying malignant cells by inducing cytotoxic reactions which result from interaction of light with photosensitive endo- or exogenous compounds, often preferentially accumulating in the target tissues. This concept led to development of several oncological treatments, for example, in dermatology, otorhinolaryngology, gastrology, ophthalmology and gynaecology.

In urology, where the main indication for PDT is multirecurrent superficial bladder cancer resistant to BCG treatment, PDT has received only marginal interest because the first generation photosensitizers did not localize with sufficient selectivity in neoplastic tissues and induced skin photosensitivity after systemic administration. Recently, interest in PDT of bladder cancers has been renewed by demonstration of the selectivity of protoporphyrin IX (PpIX) induced after instillation of 5-aminolevulinic acid (ALA). PpIX is an intermediate of the cycle of heme synthesis (fig. 1) and its intracellular content can be significantly increased when the regulatory step of the cycle is bypassed by exposing the tissue to a precursor, for instance 5-aminolevulinic acid (ALA).[4] In addition. Pp IX accumulates at much higher concentrations in malignant than in normal cells due to the reduction of ferrochelatase and iron deficiency in tumors.[5]

The results obtained by PDT in skin tumors[6] suggested that a similar approach might be used in urology. While the diagnosis of CIS and DYS is difficult or impossible during cystoscopy using white light, fluorescence cystoscopy after intravesical administration of 3% ALA solution often allows us to detect and define with precision the limits of DYS and CIS.[7,8] The sensitivity and specificity of photodynamic detection (PDD) approach 80%. The preferential accumulation of PpIX in the transformed urothelium,[9] the intravesical tolerance of ALA solutions adjusted to physiological pH values, and the absence of systemic effects reinforce the interest of such an approach.

A complete destruction of a tumor by PDT critically depends on a sufficiently high concentration and homogeneous distribution of PpIX in the malignant cell layers.[10] Although relatively high ALA concentrations were instilled into the bladder for many hours, fluorescence microscopy showed a rather irregular distribution of PpIX within superficial tumors of the bladder.[11] Also, the conditions for reaching the threshold of phototoxicity in the urothelium are not exactly known. This is not surprising as a double charged molecule like ALA is not expected to penetrate with ease across cell membranes and interstitial spaces. More lipophilic derivatives of ALA are expected to be more favored from this point of view. After traversing the cellular membrane non-specific esterases will reduce such compounds to 5-ALA. Dimethylsulfoxide (DMSO) and desferrioxamine (DES) have been

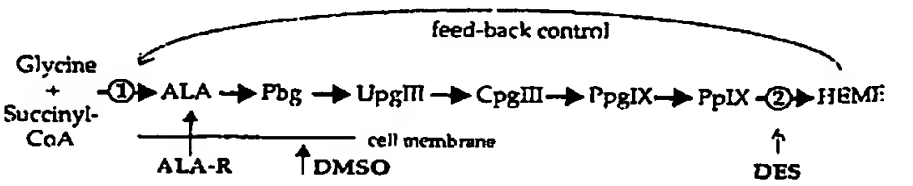

FIG. 1. Simplified scheme of heme biosynthesis and interventions used. ALA: 5-aminolevulinic acid; ALA-R: esters of ALA; Pbg: porphobilinogen; UpgIII: uroporphyrinogenIII; CpgIII: coproporphyrinogenIII; PpgIX: protoporphyrinogen IX; PpIX: protoporphyrin IX. 1: ALA synthase; 2: ferrochelatase + Fe^{++}. DMSO: dimethylsulfoxide; DES: desferrioxamine. Gray arrows: inhibitory effects.

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found to enhance PpIX accumulation. DMSO increases the transmembrane passage of small molecules.[12] DES, chelates the intracellular iron and hence inhibits the ferrochelatase activity. Consequently, PpIX is not converted into heme (fig. 1) and accumulates in cells.[5]

Thus, to define standardized and optimal conditions for PDD and PDT, a systematic study of the penetration of ALA derivatives into cells, the kinetics of PpIX accumulation, intra-urothelial PpIX distribution and mechanisms of cell photodestruction is mandatory. As a first approach to this problem, we developed an experimental setup allowing us to answer some of these questions by using bladder mucosae explanted in vitro. Here we present the kinetics and tissue profiles of intracellular PpIX accumulation, and data about phototoxicity obtained in pig or human urothelium exposed to ALA, its esters, and ALA with DMSO or DES.

MATERIALS AND METHODS

Preparation of bladder mucosa. The study required living urothelium obtained under controlled conditions. Porcine urothelium resembles human urothelium structurally[13] and can be obtained easily and reproducibly. Porcine bladders were excised from slaughtered animals. Pieces of human bladder wall were obtained from patients undergoing radical cystectomy for advanced carcinoma (3 males, 1 female; average age 73 ± 6 years). Resected bladders were opened and de visu normal and flat areas were taken for experiments. All these manipulations took about 45 minutes. Normality was confirmed by histology. The protocols were approved by the state commissions controlling animal experiments and clinical research.

The tissues were stored at 4C in Tyrode solution. The urothelium was microdissected from the bladder wall using fine scissors. The plane of cleavage passed as near as possible to the basal membrane so that thin sheets of urothelium with

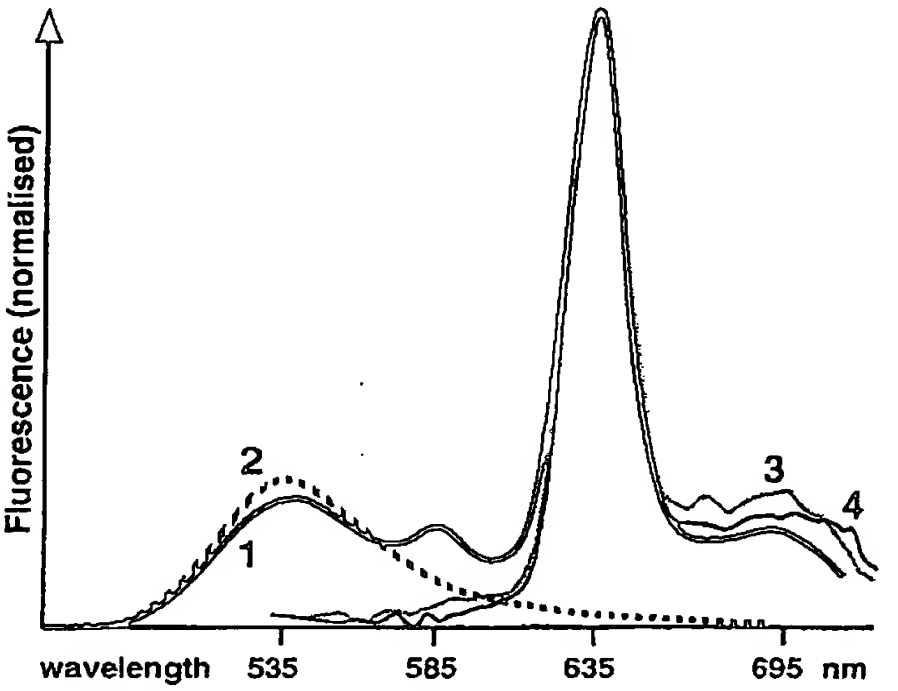

FIG. 3. Specificity of fluorescence signal. Four emission spectra as obtained from living mucosae and from frozen sections of urothelium. 1, living mucosa preincubated for 2 hours with ALA. 2, same mucosa after 10 minutes illumination. 3 and 4, urothelial sections incubated with ALA+DES and H-ALA, respectively. Spectra 1, 3 and 4 show identical peaks around 635 and 690 nm corresponding to PpIX accumulated in urothelium. In spectrum 2, PpIX signals disappeared leaving only tissue autofluorescence. Note that emission peak at 670 nm of curve 3 is due to photooxidation products of PpIX.

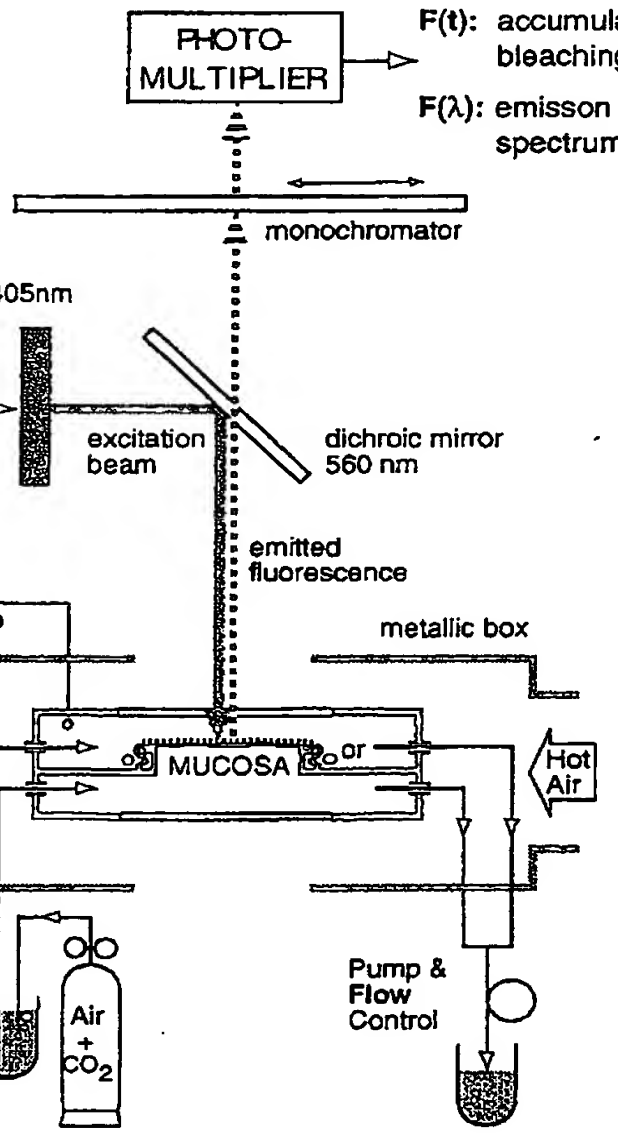

FIG. 2. Microspectrophotometry of protoporphyrin IX in bladder mucosa. Bladder mucosa is placed over and around circular rim of perspex plate and fixed with thin silicone O-ring (or). Preparation is mounted into transparent chamber and incubated in presence of ALA derivatives. Mucosa is periodically excited (100W mercury lamp, Eppendorf filter 405 nm (FWHM: 12 nm), 200 msec exposure, 45 ± 5 μW/0.05 mm.^{2}) and fluorescence emitted by cells is recorded by using EMI 20 photomultiplier. Motorized monochromator (continuous interference filter Veril, Leitz) allows analysis of emission spectrum.

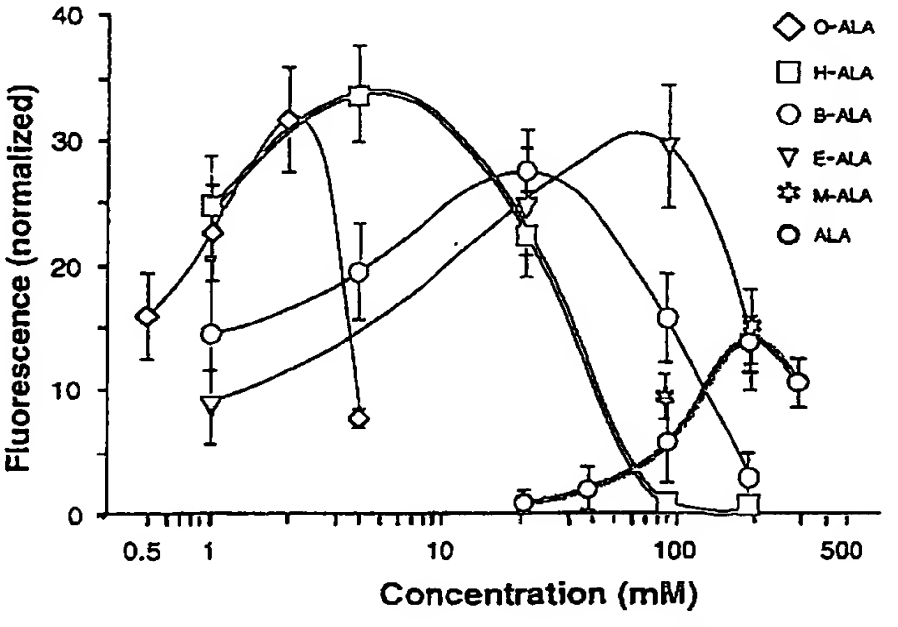

FIG. 4. PpIX kinetics for different precursors. O-ALA and H-ALA are most efficient precursors tested: at concentration 100 times lower than that of ALA, they induce 2 to 3 times higher PpIX accumulation. Determinations are made after 1 hour of incubation. Values are means ± S.D. from 20 measurements (4 mucosae per condition, 5 measurements in each mucosa). Values for M-ALA, determined at 90 and 180 mM only, were 9.4 ± 1.8 and 15 ± 3 respectively.

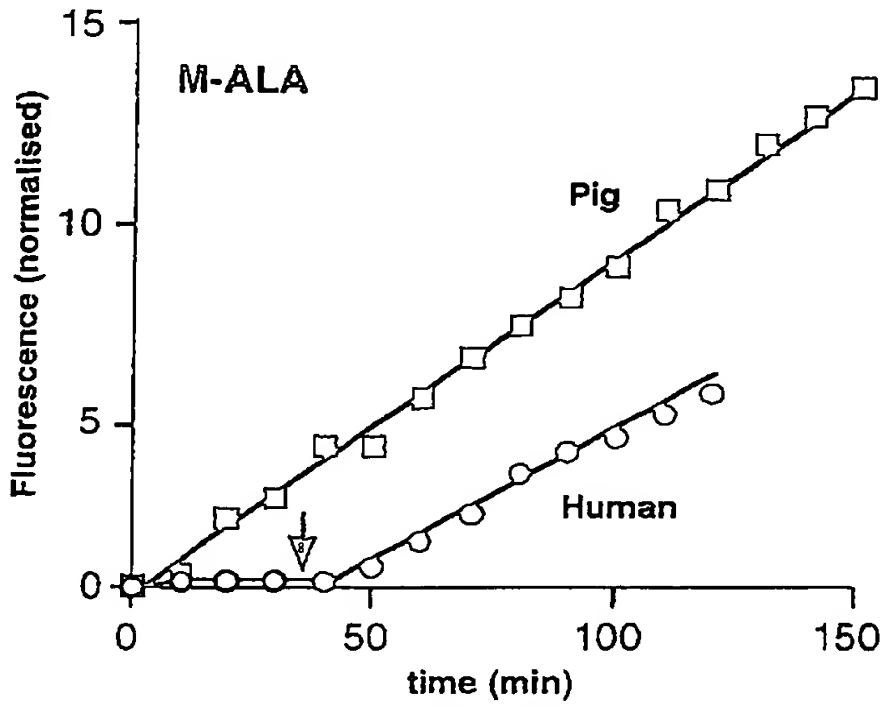

FIG. 5. Comparison of PpIX fluorescence in pig and human mucosae. During first 3 hours of incubation fluorescence increase is nearly linear in pig mucosa. Pig mucosa accumulates PpIX faster ($F = 0.082$·min., $r^2 = 0.99$) than human mucosa ($F = 0.062$·min., $r^2 = 0.98$). PpIX fluorescence is only observed when human mucosa is warmed up. M-ALA was administered at time 0. Arrow: warming of human mucosa; empty circles: human mucosa at 23C; full symbols: mucosae at 36C.

remnants of lamina propria connective tissue were obtained. These were cut into 7 × 7 mm. fragments which were mounted (urothelium up) in a transparent culture chamber designed for epithelia[14] as illustrated in fig. 2. The mucosa divided the chamber into superior and inferior compartments (diameter 20 mm., height 3 mm.) and the area exposed to exchanges was 0.125 cm.^{2} The chamber was fixed onto the plate of an epi-illumination microscope (Leitz Orthoplan) and thermostabilised at 36 ± 0.5C. The inferior compartment was continuously perfused by oxygenated Tyrode solution. Solutions of ALA derivatives were injected as a single dose into the superior compartment.

Media. The Tyrode solution contained (in mmol./l.): 143.0 Na^+, 2.0 K^+, 0.8 Mg^{++}, 1.4 Ca^{++}, 122 Cl^-, 20.0 HCO_3^-, 3 $H_2PO_4^-$, 1.2 SO_4^-, 8 glucose (osmolarity 290 mOsm/l.) and was saturated with air enriched with 5% CO_2 (pH 7.5). ALA and some of its derivatives were dissolved in phosphate buffer saline at 4C, and the pH was adjusted to 5.2 ± 0.5. Dimethylsulfoxide (DMSO) (17.5 μM) and desferrioxamine mesylate (DES) (15 μM) were added to some solutions. All solutions were colorless. They were stored on ice and used within one hour.

ALA was from Merck (Dietikon, Switzerland), methyl-ester (M-ALA) and DES were from Fluka (Buchs, Switzerland). Ethyl-(E-ALA), butyl-(B-ALA) hexyl-ester (H-ALA) and octyl-ester (O-ALA) were synthesized.[15] Their purity was superior to 95%.

Spectrofluorometry. The kinetics of urothelial PpIX accumulation with respect to precursor concentration and to time of administration were characterized as follows (fig. 2). The urothelium, incubated with a given precursor, was excited by violet light (405 nm, 45 ± 5 μW/0.05 mm.^{2}, 200 ms) each 10 minutes or each hour and the fluorescence emitted by the cells, which is taken to be proportional to the cell PpIX concentration, was passed through a low pass filter (>610 nm) and recorded by a photomultiplier. The specificity of the fluorescence signal was systematically checked by analyzing the emission spectra.

The spatial distribution of PpIX across the mucosa was determined at selected time intervals in serial 25 μm. thick frozen sections. To avoid strong photobleaching due to light exposure, the samples were prepared in the dark. The profiles of PpIX fluorescence within the mucosa were determined by scanning the fluorescence signal across the section.

Cell viability. At the end of experiments, the urothelium was exposed to acridine orange (dissolved in Tyrode 1:10000) which stains nuclei of living cells only. The proportion of labeled nuclei was evaluated by fluorescence microscopy (excitation at 405 nm, emission> 560 nm). In some cases, the time-course of PpIX photodestruction (photobleaching) was determined and the consequent phototoxicity effects on urothelial cell were studied by using electron microscopy. Two hours after the exposure to light, the mucosae were fixed in paraformaldehyde/glutaraldehyde and embedded in Epon. Sections of 700 Å were analyzed by transmission microscopy (Zeiss, Germany). Some mucosae were dehydrated and dried (CPD 030 critical point dryer, Balzers, Liechtenstein), coated with 300 nm gold (S150 sputter coater, Edwards, Zivy, Basle) and studied by using scanning electron microscope (JEOL, Tokyo).

Statistical analysis. Supposing that the photobleaching of endogeneous chromophores is small, fluorescence values were normalized, that is, corrected for the tissue autofluorescence [$I_n(t) = (I_0(t)\text{-}AF)/AF$)]. The data are presented as arithmetical means and standard deviations. A paired bilateral t test was used to compare the results and values of $p \leq 0.05$ were considered as significant.

Pp IX accumulation in urothelium in response to ALA derivatives

Precursor	Hours of Administration						
	1	2	3	4	5	6	7
ALA	17 ± 7	31 ± 7	41 ± 6	54 ± 9	64 ± 7	67 ± 10	65 ± 12
ALA+DMSO	14 ± 3	36 ± 8	57 ± 17	75 ± 17	90 ± 18	90 ± 18	87 ± 31
M-ALA	15 ± 3	35 ± 5	53 ± 5	69 ± 9	82 ± 13	94 ± 14	91 ± 20
M-ALA+DMSO	18 ± 3	42 ± 5	68 ± 9	79 ± 11	97 ± 14	115 ± 18	114 ± 17
ALA+DES	21 ± 2	48 ± 11	81 ± 13	117 ± 14	157 ± 36	193 ± 50	256 ± 27
B-ALA	27 ± 3	61 ± 7	100 ± 14	134 ± 26	163 ± 28	156 ± 27	144 ± 25
H-ALA	34 ± 4	67 ± 6	105 ± 12	134 ± 20	167 ± 27	175 ± 26	189 ± 37
H-ALA+DES	32 ± 3	62 ± 5	98 ± 4	126 ± 10	153 ± 7	172 ± 8	170 ± 16

Values of normalised fluorescence are given as means ± standard deviations. Data were obtained from 4 mucosae per condition, and from 5 regions in each mucosa. ALA: δ-aminolevulinic acid, M-: methyl, B-: butyl, H-: hexyl, DMSO: dimethylsulfoxide, DES: desferrioxamin mesylate. Upper frame: values different from ALA at $P \leq 0.05$; lower frame: values different from ALA at $p \leq 0.01$.

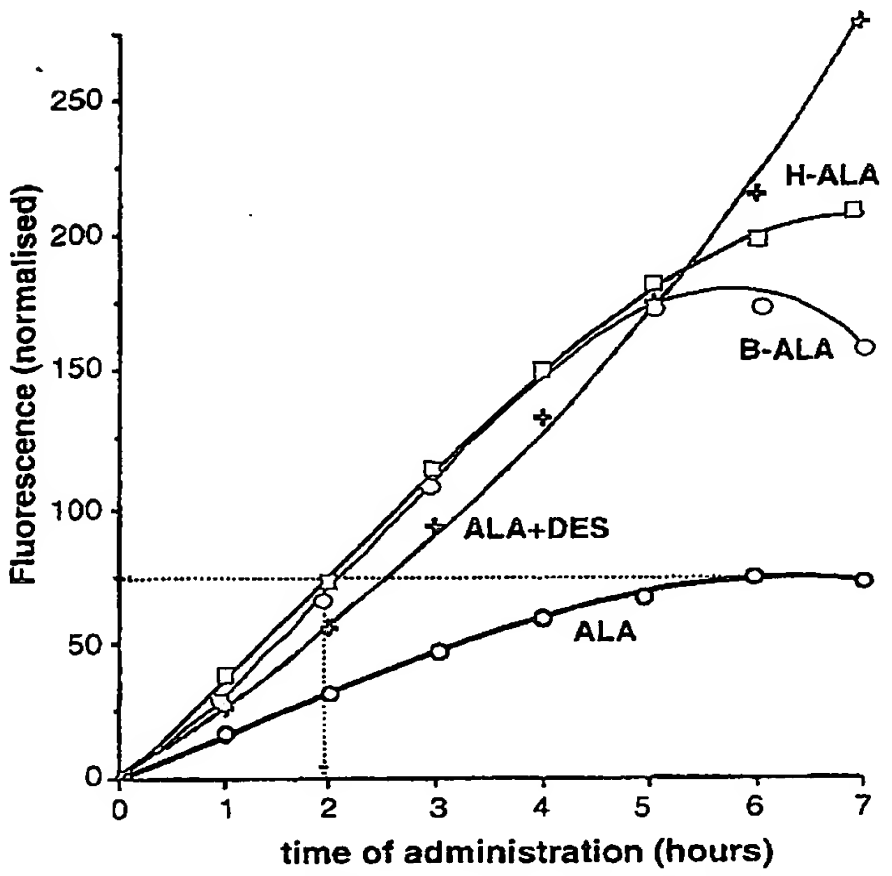

FIG. 6. PpIX accumulation with ALA, ALA+DES, B-ALA and H-ALA. H-ALA, although administered at much lower, and hence less toxic concentration, can considerably shorten time of administration (dotted lines), allowing both rapid and efficient PDD and PDT. (ALA and ALA+DES: 700 mOsm; H-ALA: 290 mOsm). Values are means extracted from table.

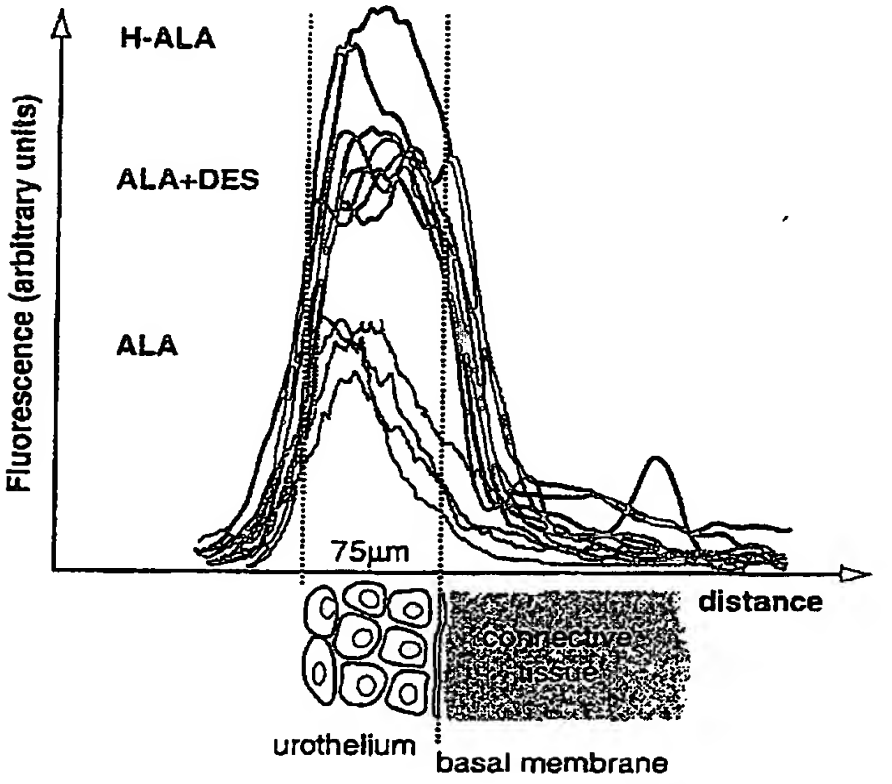

FIG. 7. Fluorescence intensity as measured across bladder mucosa. H-ALA allows highest and most homogeneously distributed PpIX accumulation in urothelium. Data from 12 fluorescence scans across mucosal sections were corrected to mean urothelial thickness. Scanning speed 100 mm./sec.; excitation at 405 mm.; width of illuminated slit 30 µm.; emission at 610 nm).

RESULTS

Nature of the fluorescence signal. The spectral analysis of the light emitted by the mucosa shows that, in the absence of PpIX precursors, the tissue emits weak autofluorescence giving a very small signal in the red domain (≥610 nm). This

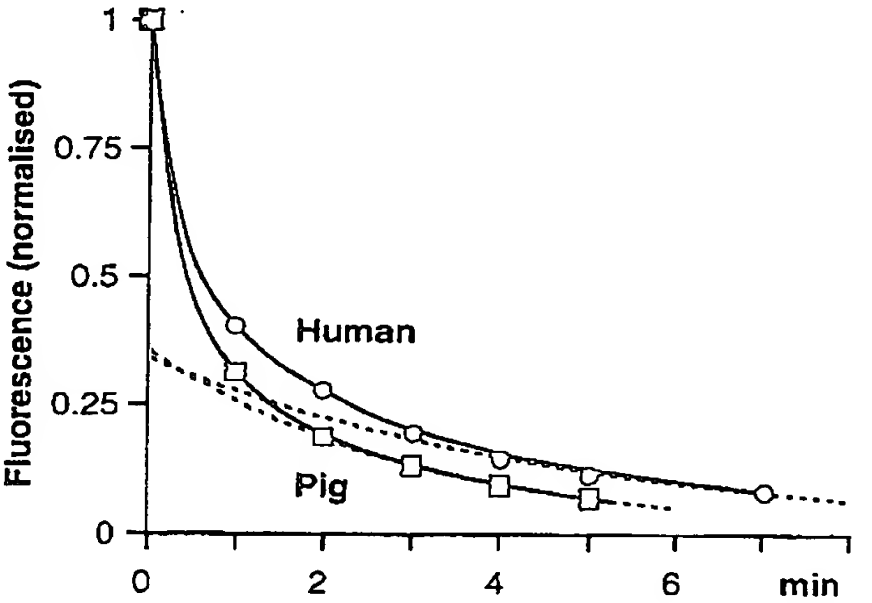

FIG. 8. Photosensitivity of fluorescence signal. Time-course of photodestruction of PpIX upon continuous illumination (405 nm, 4.75 J/cm.^{2}) of mucosa. Photobleaching appears to show fast and slow phases. Residual signal corresponds to autofluorescence.

signal serves to normalize the specific PpIX fluorescence. The latter appears in presence of PpIX precursors as one major (635 nm) and one minor (690 nm) peak (fig. 3). No differences were observed between the emission spectra shape of pure ALA- and ALA-esters-induced PpIX. The PpIX peaks disappear after exposure of the mucosa to light.

Effect of PpIX precursor concentration. Fig. 4 illustrates the results obtained for ALA and its esters after 1 hour of incubation. All precursors show similar kinetics: with increasing concentration, the fluorescence intensity increases, reaches a maximum and then decreases sharply. As compared with ALA, the applied concentrations of E-ALA, B-ALA, H-ALA and O-ALA are respectively 2, 10, 45 and 90 times lower, but nevertheless result in 2 to 2,5 times higher fluorescence.

The reduction of fluorescence at high concentrations may indicate significant cytotoxicity of ALA esters which resulted in peeling of the mucosa (as with ALA at 180 mM), or even immediate cellular lysis (as with H- and B-ALA at 180 mM)(not shown). Due to its higher lipophilicity, precipitation of O-ALA in aqueous solutions at high concentrations may reduce the total drug content.

Accumulation of PpIX in the mucosa. Both the pig and human mucosae exposed to precursors accumulate PpIX, after warming to 37C, that is, upon metabolic activation. This is illustrated in fig. 5 which shows an example of human mucosa with an accumulation rate comparable to that of pig mucosa. However, on the average, the human mucosae (n = 4, 3 males, 1 female, mean age 52 years) accumulated PpIX 3.6 times less than the pig mucosae.

The table shows the results obtained in the pig mucosae with ALA derivatives used at their respective optimal concentrations. In all cases, the fluorescence increased nearly linearly up to four hours and saturated between the 6th and 7th hours. With ALA+DES, the fluorescence continued to increase exponentially up to 7 hours. ALA was the least efficient of the tested precursors. The other substances induced a significantly faster and greater (1.3 to 3 times) increase of PpIX fluorescence. H-ALA and ALA+DES were the most efficient, but H-ALA and B-ALA were shown to reach the highest fluorescence at the shortest administration times (table, fig. 6). No significant difference in PpIX formation was observed between H-ALA and B-ALA used at their optimal concentrations. This indicates that the biosynthetic pathway of heme was saturated by the more lipophilic esters, while PpIX production induced by pure ALA never reached sufficiently high intracellular drug contents.

Iso-osmolar replacement of sodium in the Tyrode solution by choline did not modify the accumulation kinetics of PpIX (not shown) indicating that the penetration of ALA derivatives into the cell does not involve a sodium-dependent co-transport.

At the end of each experiment (24 mucosae, 7 hours of application, 8 precursors at their optimum concentration), upon labeling with acridine orange, all preparations showed cells with bright nuclear fluorescence, indicating that the urothelium remained alive.

Distribution of PpIX across the mucosa. The fluorescence profiles across the mucosae were recorded after 2 hours of incubation with ALA, H-ALA and ALA+DES. As the thickness of urothelium varied (from 66 to 88 μm.), the results are presented after a homothetic translation to 75 μm. As shown in fig. 7, PpIX fluorescence induced by the 3 precursors is limited essentially to the urothelial cells. With ALA, the fluorescence is limited mostly to the superficial cells while with ALA+DES and especially H-ALA, the fluorescence is about twice as high and distributed in all urothelial layers.

Phototoxicity. If the mucosa accumulating PpIX is exposed to continuous violet illumination for 10 minutes, the urothelial fluorescence decays. Supposing an exponential decay with time, the two rate constants are of about 30 seconds and 3 minutes (fig. 8). This time dependence of the fluorescence signal may be due to more stable photoprotoporphyrins formed by photodegradation of PpIX. After 10 minutes of illumination, the specific fluorescence is no longer detectable which indicates that most fluorescing porphyrins were destroyed.

When the mucosae illuminated for 10 minutes were incubated for 2 additional hours, the cells that had been exposed to light died. This was documented by electron microscopy (fig. 9) which revealed damaged mitochondria, marginalisation of nuclear chromatin, vacuolized cytosol and fenestration of the plasma membrane. The superficial cells were rounded and lost contact with each other. In mucosae preincubated for shorter times (for example, ALA, 2 hours), the necrotic changes were found mostly the in the superficial cells. In mucosae preincubated for longer times (for example, ALA, 6 hours) the urothelial necrosis was complete while the underlying connective tissue was not damaged. The necrosis

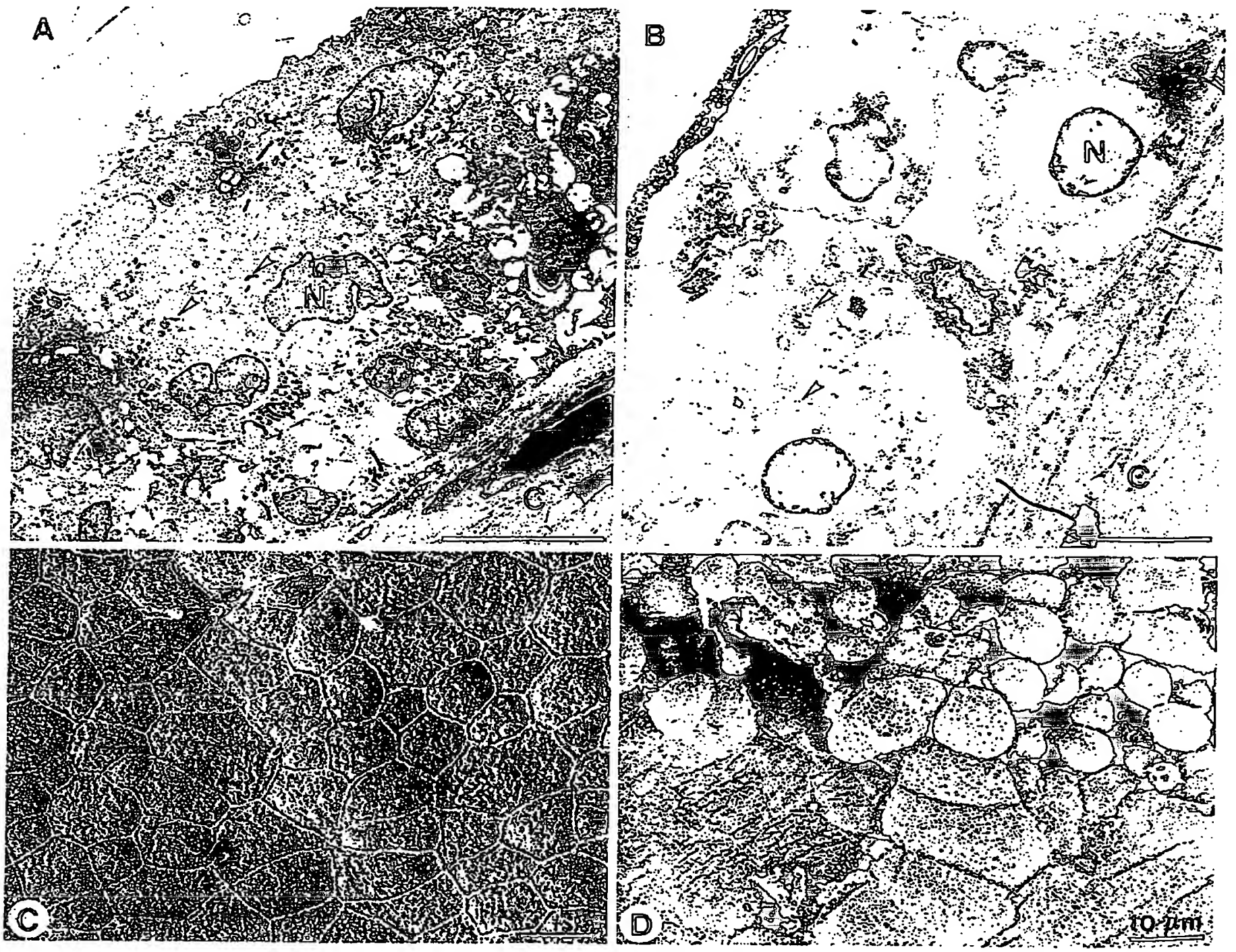

FIG. 9. Urothelial necrosis induced in bladder mucosae by exposure to light. Transmission (*A, B*) and scanning (*C, D*) electron micrographs of pig mucosae incubated for 6 hours with ALA. *A, C,* control mucosae with normal intra- and intercellular structure. *B, D,* mucosae exposed to light (405 nm, 4.75 J/cm.^{2}, 10 minutes) and incubated for 2 additional hours, showing marginalized chromatin, swollen mitochondria, vacuolized cytoplasm, fenestrated plasma membrane and lost intercellular contacts. Arrows: mitochondria, N: nuclei, C: connective tissue. Bars: 10 μm.

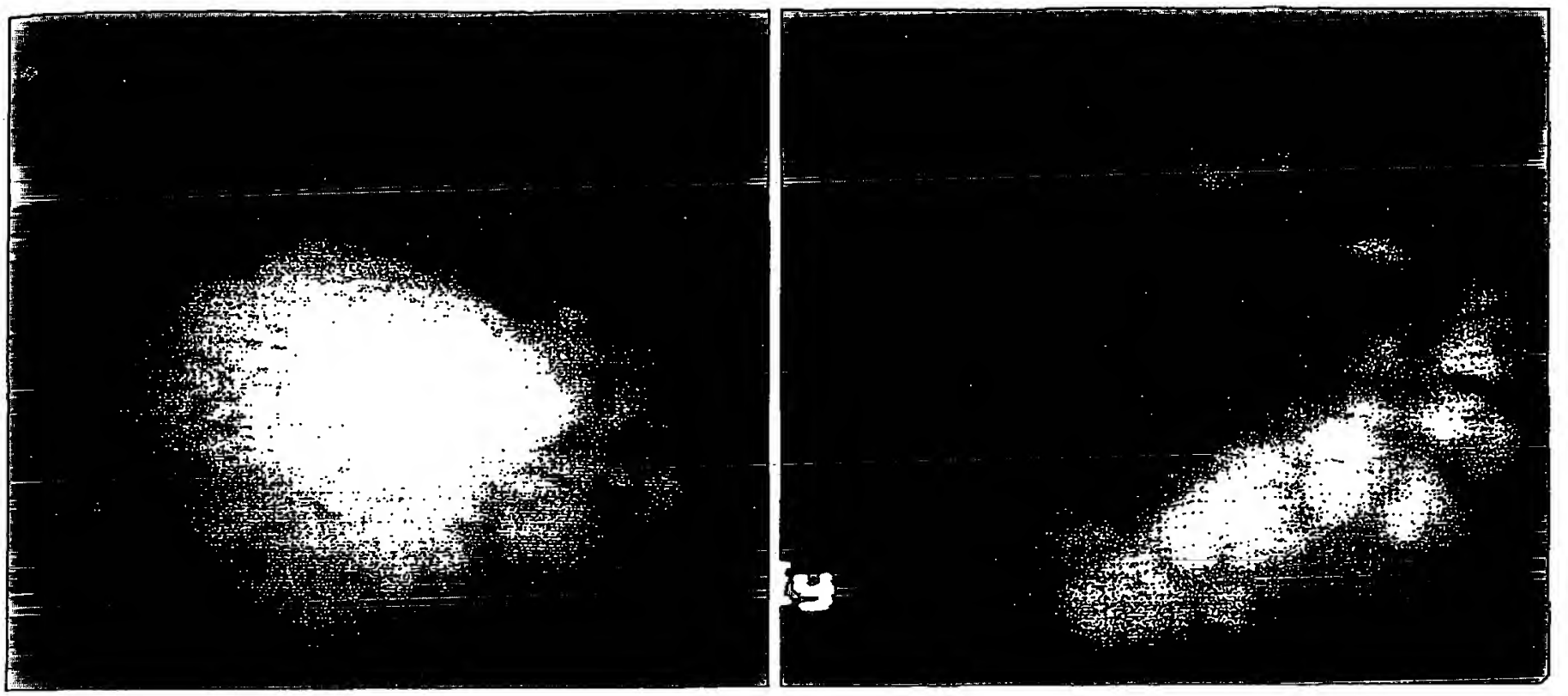

FIG. 10. Endoscopic view of human bladder papilloma. Left: image in white light. Dimension of tumor is 4 × 8 mm. Right: image in blue-violet light after instillation of hexyl-ALA (8 mM) for 2 hours. Highly fluorescent tumoral cells (red) appearing on background of non-fluorescent (green) normal tissue. Histological diagnosis: pTaG1. (With permission.[24])

induced by violet light was confined to the illuminated area and was surrounded by normal cells (fig. 9).

DISCUSSION

The use of bladder mucosa explanted into a superfusion chamber is a powerful tool which, unlike cell cultures, permits the study of epithelia with intact architecture and functional polarity. This is especially valuable for evaluation of penetration of substances into the normal as well as pathological epithelia. Although the blood circulation is eliminated in this preparation, the renewal of submucosal medium secures the homeostasis and survival of urothelial cells.

Urothelium of pig and human origins has a very similar structure although surface proteoglycans are not identical and mucus cells are absent in the normal human bladder.[13] In our hands, both mucosae also show similar accumulation kinetics although the final fluorescence intensity in the human case is lower. This reflects possibly decreased cell viability due to unavoidable and long (about 2 hours) hypoxia resulting from early vascular ligation during the bladder operations.

The synthesis of supplemental PpIX must be preceded by penetration of the precursor across the plasma membrane. Three results strongly suggest that the precursors penetrate into the cells by simple diffusion: 1) esters with the longer aliphatic moiety (hence more lipophilic) penetrate faster and to a greater extent, 2) similar kinetics of PpIX formation for H-ALA (4 mM) and B-ALA (20 mM) as well as comparable fluorescence levels under optimized concentrations suggesting a passive, concentration gradient driven uptake as predicted by simple diffusion laws, and 3) absence of sodium in the solutions does not decrease the PpIX accumulation as would be expected for a sodium-linked co-transport frequently operating for amino-acid cell transport. This is in agreement with results obtained in cell cultures.[16]

Once inside the cell, the esters of the ALA are hydrolyzed by non-specific cell esterases and free ALA appears in the cytoplasm.[16] The cell fluorescence will, however, increase only upon metabolic activation in the mitochondria, which confirms that PpIX synthesis is an energy-dependent process (fig. 5).

Whatever the precursor used, the time profiles of accumulation are similar: with increased concentration, the synthesis of PpIX increases to a maximum and then decreases to zero. Similar results were obtained in cell cultures.[16] In our case, the final decrease is accompanied by a loss of cell adherence and presence of free cells floating in the superfusate. This might be due to the hypertonicity of the solutions[17] (fig. 4) and/or to the toxicity of ALA itself. Indeed, the cells also peel off in presence of diluted but highly penetrant precursors such as H-ALA where the resulting high cytoplasmic ALA concentration might favor the production of oxygen reactive radicals[18] and subsequent cell injury.

PpIX fluorescence increases essentially linearly up to 4 hours and then attains a plateau value or even slightly decreases. Similar results were found in cell cultures from tumoral bladders and in rat urothelium in vivo.[19–21] It is possible that this plateau reflects not only the balance between PpIX synthesis and PpIX utilization, which should happen with all precursors, but also the penetration of precursors into deeper lying cells, which should increase with liposolubility of the ALA esters, and appears to be shown by the fluorescence profiles. The observed dependence of decreasing optimal concentration with increasing chain length of the ALA derivatives has been confirmed in cell cultures.[16] O-ALA at higher concentrations shows a tendency to precipitate at physiological pH values which limits its clinical use.

Clearly, DMSO, which is supposed to increase the membrane permeability, potentiates the PpIX accumulation under our conditions much less than the esterification of ALA. DES, a selective an iron chelator, when combined with ALA, inhibited the conversion of PpIX to heme and thus potentiated PpIX accumulation to higher levels and before a plateau was reached. This suggests that the synthesis of PpIX in itself was not slowed down in any of the conditions used. Surprisingly, DES showed no potentiation when combined with H-ALA. The explanation of this result awaits new experiments.

The photobleaching curves show a fast and a slow compo-

nent, which might result from the bleaching of fluorescent photooxidation products, originating in different intracellular compartments.[22] A mixture of isomeric chlorins resulting from the first photodegradation process of PpIX has been shown to be about 10 times more stable to photo-oxidation than PpIX.[23] Their appearance may be seen from the fluorescence emission peak at 670 nm (fig. 3; curve 3). Our results show that 10 minutes exposure to light is sufficient to induce cell necrosis in all layers of a normal urothelium. In vivo records of photobleaching could help to dose the radiation energy necessary to destroy the tumoral tissue.

CONCLUSIONS

The in vitro preparation of bladder mucosa developed in this work has brought additional valuable information on the dynamics of accumulation and destruction of photosensitive molecules used in the PDD and PDT of urothelial carcinoma. In the case of PpIX, H-ALA seems to be a good compromise between lipophilicity, solubility and performance with respect to high PpIX formation and low precursor concentration. In comparison with ALA, it increases and accelerates the PpIX synthesis, penetrates into all epithelial cell layers, and is efficient at low concentrations. At these low concentrations it preserves urothelial viability and allows effective cell photodestruction. The optimal time necessary for PDD and PDT at the conditions applied is shortened from near 6 to 1.5 hours. As shown in fig. 10, under these conditions applied in humans, PDD results confirm the predictions and show selective accumulation of PpIX in urothelial carcinoma.

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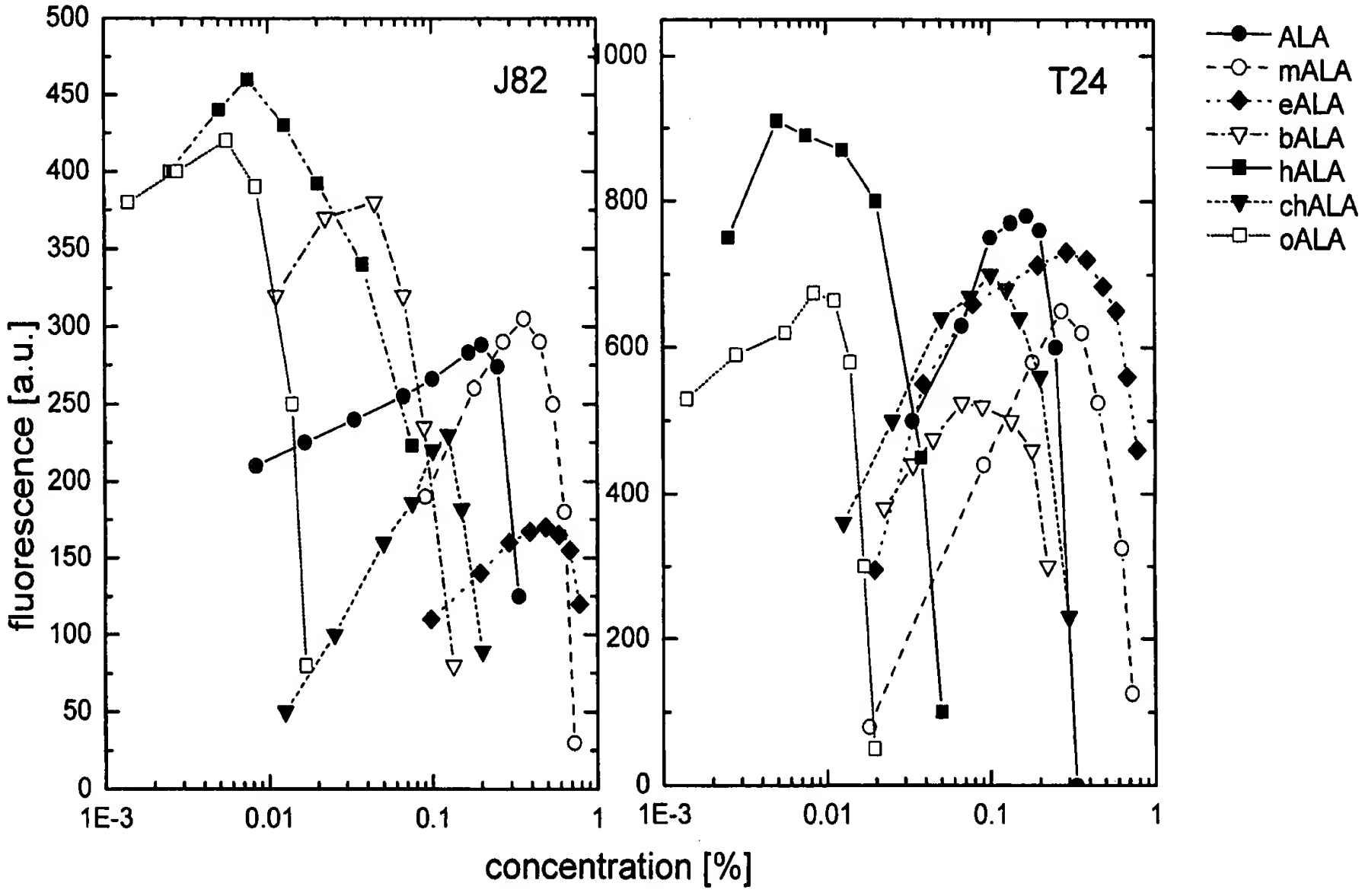
PPIX production measured by fluorescence spectroscopy in Vitro for different precursors



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DOSE-RESPONSE OF DIFFERENT ALA-ESTERS ON THE PIG BLADDER MODEL

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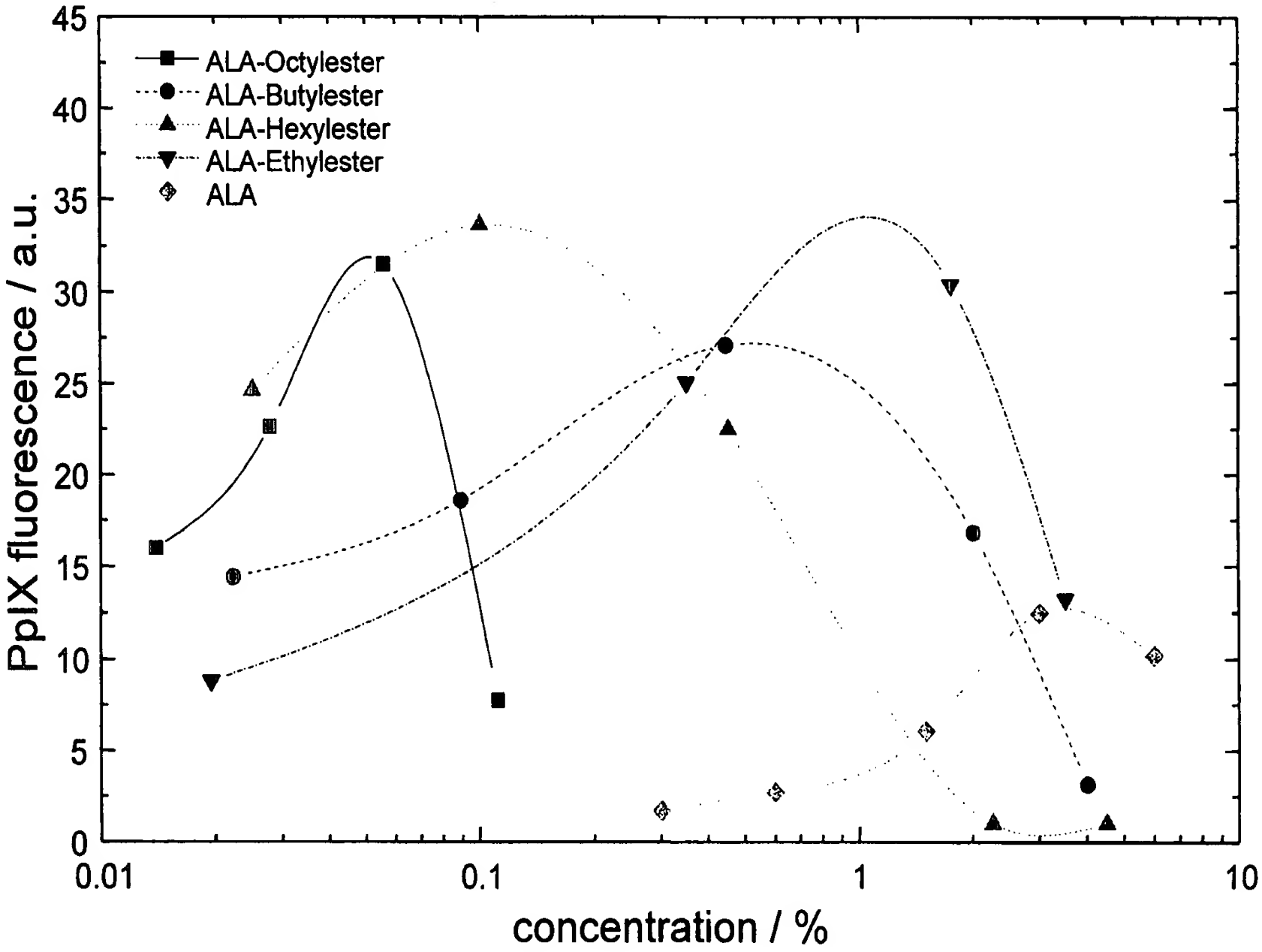

CLINICAL SPECTROSCOPY MEASUREMENT OF h-ALA-INDUCED PPIX AS COMPARED TO ALA in vivo IN A pTaG2 CANCER (4 hours instillation + 2 hours resting time)